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TITLE: Defining the Dormant Tumor Microenvironment for Breast Cancer Prevention and Treatment

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14. ABSTRACT

Tamoxifen and parity induce ECM remodeling resulting in microenvironments that are anticipated to be inhibitory to breast cancer progression. Given the technical difficulties in studying tumor cell dormancy in the context of secondary lesions, we propose to use suppression at the primary site as a model for breast cancer dormancy. **Aims:** 1) Identify ECM proteins of the quiescent mammary gland. 2) Determine whether a *reconstituted* 'tumor suppressive' ECM causes tumor cells to enter a quiescent/dormant-like state *in vitro*. 3) Validate the 'tumor suppressive' rat ECM signature in breast tissue of parous women and in women with anti-estrogen treatment compared to controls using IHC assays. **Results:** Mammary ECM isolated from parous rats suppressed tumor progression in *in vitro* and *in vivo* assays, and supported adherens junction formation. Mass spec-based proteomics and label-free quantitative analysis identified an increase in collagen I in parous compared to nulliparous rat mammary glands; data that contradicts the paradigm that higher fibrillar collagen density is tumor promotional. Analysis of collagen architecture revealed an increased amount of anisotropic collagen fibers in parous glands relative to nulliparous glands, suggesting reduced tensional properties with parity. **Impact on Breast Cancer Research/ Patients:** Objective: identify ECM proteins that support tumor cell dormancy for the purpose of developing cell culture conditions suitable for screening drugs targeting dormancy. This work has the potential to yield high impact data and open new avenues of investigation into mechanisms and targeting of tumor dormancy.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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TABLE OF CONTENTS:

INTRODUCTION: Whether the microenvironment can actively inhibit tumor progression has not been well studied. It is known that tumor cells can remain at primary sites or arrive at secondary sites in high numbers but fail to expand and further, that micro-tumors and solitary tumor cells can reside in a dormant state for decades. Data from numerous studies strongly suggest that extracellular matrix (ECM) proteins can impart a dormant suppressive phenotype onto malignant cells. We hypothesis that insight into tumor cell dormancy can be obtained from studying the response of the mammary stroma to tamoxifen treatment and parity, two conditions that protect the epithelium from tumorigenesis. Specifically, we propose that tamoxifen and parity induce ECM remodeling resulting in microenvironments that would be inhibitory to breast cancer progression. Given the technical difficulties in studying tumor cell dormancy in the context of secondary lesions (literally searching for a needle in the haystack), we propose to use dormancy at the primary site as a model to investigate the role of ECM in breast cancer dormancy. The objective of this BCRP Idea Award is to identify ECM proteins that support tumor cell dormancy. Proteomic analyses of ECM proteins have been severely hindered by the insoluble and digestion resistance nature of ECM. Our lab has explored the utility of combining rapid ultrasonication and surfactant assisted digestion for the detailed proteomic analysis of ECM samples. When compared to traditional overnight digestion, this optimized method dramatically improved the sequence coverage for ECM proteins. Using this optimized proteomic protocol, we will Identify ECM proteins of the quiescent mammary gland. Determine whether a *reconstituted* „tumor suppressive" ECM causes tumor cells to enter a quiescent/dormant-like state *in vitro*. And validate the „tumor suppressive" rat ECM signature in breast tissue of parous women and in women with anti-estrogen treatment compared to controls using immunohistochemical assays.

BODY:
STATEMENT OF WORK AND ACCOMPLISHMENTS
Task 1: Generate age-matched nulliparous and parous rats to obtain mammary tissue for ECM isolation.
a) Randomize female Sprague Dawley rats, 80 days of age, into nulliparous and parous groups. Each group will include 12 rats.
b) Breed rats in the parous group. After parturition, normalize the number of pups to eight. Ten days after parturition remove the pups to initiate involution.
c) Mammary glands will be harvested one month post weaning (fully regressed) from parous rats and from age-matched nulliparous rats. Vaginal smear will be taken prior to euthanasia to determine the stage of estrous cycle. This information may be important to explain variation in mammary gland biology among animals in same group. To confirm the data from vaginal smear, cervical tissue will be collected and processed for histological evaluation.
d) Inguinal mammary glands (4-6) will be dissected: the mammary gland portion with lymph nodes will be excised and fixed in formalin for IHC, while the remaining portion of the glands will be snap frozen.
e) Frozen, lymph-node free mammary tissue from tamoxifen-treated rats are available for these studies (injected s.c. daily with1 mg/kg body weight tamoxifen (Sigma) dissolved in ethanol and suspended on seasame oil and vehicle controls)
f) Mammary ECM will be isolated from lymph-node free inguinal mammary glands using a protocol published by Dr. Schedin"s lab.
Progress in first year of funding-task 1 completed.

Task 2: Characterize prominent ECM proteins in nulliparous, parous, and tamoxifen-induced mammary glands using mass spectrometry.
a) Digest nulliparous, parous, and tamoxifen-induced mammary ECM using in-solution ultrasonication assisted tryptic digestion (UATD) method. ==Parity arm completed.==
b) Analyzed the digested mammary ECM samples from nulliparous, parous, and tamoxifen treated groups by mass spectrometry. To improve peptide abundance, we will use strong cation exchange fractionation for mammary ECM follow by LC/MS/MS analyses. MS data will be searched against IPI.Rat (v3.53.fasta) database using Protein Prospector, LC Batch-Tag Web (Version 5.0, UCSF). ==Parity arm completed.==

Task 2 progress in first year of funding
1. Proteomic analysis has been completed on parous and nulliparous matrix preps. We identified parity-related compositional differences in the mammary ECMs by performing in depth mass spectrometry-based proteomics and label-free quantitative analysis. This analysis consists of five samples for each group at three different time points to avoid any instrument bias.

        1. **Table 1** depicts the top ECM proteins identified in rat mammary gland.

        2. **Figure 1** depicts the composition of mammary ECM proteins as a pie chart, and demonstrates that collagen I, III, VI and XIV are the dominant mammary collagens, as well as the dominant ECM proteins of the mammary gland. Evidence that the mammary stroma is rich in proteoglycans is provided by these analyses as well.

Proposed task 2, year two aims:
1. Complete data analyses on nulliparous and parous matrix ECM data set.
2. Isolate mammary ECM from tamoxifen treated rats to begin protein identification on these matrices for comparison with parous mammary ECM.

Task 3: Construct „tumor suppressive" ECM signature by identifying proteins in common between tamoxifen-induced and parous mammary ECM and that differ from the nulliparous ECM protein profile.
a) Utilize the mammary ECM protein profiles from **Task 2** to determine relative protein abundance between nulliparous, parous, and tamoxifen treated groups using label-free quantitative LC-MS/MS analyses, so that both qualitative and quantitative ECM profiling data are obtained. ==Parity arm completed.==
b) Validate our data using Western Blot analyses.
Task 3 progress in first year of funding:
 1. Quantitative analyses of ECM protein abundance between nulliparous and parous glands revealed that fibrillar collagen 1 was detected at higher levels in ECM isolated from parous glands than nulliparous glands (**Fig 2a&b**), a result confirmed by western blot analysis in ECM preparations (**Fig 2c**). Evidence that collagen organization differs between groups is suggested by differences in levels of detected decorin and Col XIV (Fig **2c&d**), two known collagen crosslinking proteins [1].

2. Using two-photon microscopy to obtain second harmonic generated (SHG) imaging of collagen fibers *in situ*, we have confirmed that collagen organization in the parous gland is quantitatively different than collagen organization in the nulliparous gland (**Fig 3a**), and that further, the organization of parous collagen is consistent with a more compliant, less tumor promotional collagen structure (**Fig 3b,c&d**) [2, 3].

Proposed task 3, year two aims:
1. Validate that differences detected by mass spectrometry proteomics in collagen crosslinking proteins reflect compositional differences in isolated mammary matrices, as well as in mammary tissue from nulliparous and parous rats.
2. Evaluate collagen fiber directionality in control nulliparous and tamoxifen-induced mammary ECM via SHG imaging.
3. Perform mammary ECM isolation and protein analysis from tamoxifen treated rats.

Task 4: We will determine whether the _reconstituted_ „tumor suppressive" ECM can suppress organoid growth and ability to form complex structures in a panel of breast cancer cell lines using 3D culture model. The effects

of the reconstituted matrix will be compared to effects of the individual prominent ECM proteins. For this task, parous and tamoxifen-induced mammary ECM will serve as positive controls and „tumor supportive" nulliparous mammary ECM will serve as a negative control for „tumor suppressive" ECM.

a) Reconstitute „tumor suppressive" ECM from the prominent (top ~5 ECM) proteins from qualitative and quantitative ECM profiling data that will be obtained in **Task 3**.

b) This „tumor suppressive" ECM will be used as a substratum for the 3D culture experiments and we will evaluate its effect on tumor cell growth and organoid organization.

Task 4 progress in first year of funding:

1. Our hypothesis that the parous mammary gland ECM would be tumor suppressive in comparison to the nulliparous gland ECM is based on the premise that the parous mammary gland would be suppressive to growth of mammary tumor cells. This project was in fact "high risk" because in fact, only one previous publication has demonstrated that the parous mammary hosst is tumor suppressive in comparison to the nulliparous host. These data were obtained in a chemical carcinogenesis model, and did not distinguish between local mammary and systemic effects of parity [4].

We first performed xenograft, proof of principal studies where MCF10DCIS.com or D2.OR cells were injected into age-matched nulliparous and parous hosts. For both cell lines, tumor cell growth was suppressed in the parous group in comparison to the nulliparous group (**Fig4a&b).**

2. These xenograft studies demonstrate that the parous host is tumor suppressive in comparison to the nulliparous host, but do not identify a role for the mammary ECM in mediating these suppressive effects. We next performed an *in vivo* tumor study where mammary ECM from either nulliparous or parous mammary glands was pre-mixed with tumor cells prior to injection into the mammary fat pad of nulliparous mice. Prior to utilizing our mammary matrices for *in vivo* studies, we confirmed that both nulliparous and parous matrix supported the 3D organization and proliferation of normal mammary epithelial cells in 3D culture, and confirmed that the matrices did not induce cell death. As seen in **Figure 5**, immortalized but non-transformed human mammary epithelial MCF12A cells organize into complex 3D branched and acini-like structures without evidence of dying cells when plated onto Matrigel (positive control), nulliparous matrix and parous matrix. IHC analyses for cell proliferation demonstrate proliferating cells under all conditions (data not shown). As seen in **Figure 6**, mammary ECM isolated from parous mammary glands suppressed MCF10DCIS cell growth, confirming a role for mammary ECM in mediating the tumor suppressive effects of parity on tumor cells.

Proposed task 4, year two aims:

1. Continue to identify compositional differences between tumor supportive and tumor suppressive ECM so that a reconstituted ECM can be evaluated for tumor suppressive attributes in 3D cell culture.

2. Repeat the xenograft ECM study described in Figure 6A with D2.OR cells.

3. Pursue our observation that collagen structure of the parous mammary gland rather than abundance determines tumor cell invasive behavior using 3D cell culture models.

Task 5: In comparison to individual prominent ECM proteins, determine whether „tumor suppressive" ECM inhibits tumor cell motility and invasion in transwell filter assays. For this task, parous and tamoxifen-induced mammary ECM will serve as positive controls and „tumor supportive" nulliparous mammary ECM will serve as a negative control for „tumor suppressive" ECM.

a) Transwell filter assays will be used to evaluate motility and invasion. The filter membranes will be pre-coated with the reconstituted „tumor suppressive" ECM in the presence or absence of individual prominent ECM proteins or rat mammary ECM pads. Completed.

b) MCFDCIS cells, D0.R2 cells, and D2A1 cells will be overlaid on the matrices and tested for their ability to migrate across the filter membranes. Completed.

Task 5 progress in first year of funding:

1. D2OR cells as well as MCF10DCIS cells displayed low motility and invasion rates in standard transwell filter assays (data not shown). Thus, an alternative approach was selected, which was to use motility and invasion in 3D culture as our endpoint for to assess for the ability of parous matrix to suppress these metastatic phenotypes. In 3D culture, parous

matrix suppresses tumor cell invasion in two different mammary tumor cell lines (murine D2OR and human MCF10. DCIS), as detected by decrease number of invasive filapodia and single migratory cells as well as decreased phospho-ERK protein levels as detected by Western blot (**Fig 7a&b**).

Proposed task 5, year two aims:
1. Repeat phosphor-ERK Western blot for replica-data.
2. Quantitate number of invasive filapodia and single migratory cells from 3D cultures.

Task 6: In 3D culture, we will determine whether „tumor suppressive" ECM induces a dormant-like state in breast cancer cells by examining quiescence/dormancy-regulated cellular proteins.
a) Utilize the 3D culture model described in **Task 4** to generate organoids and protein lysates.
b) Examine a panel of quiescence/dormancy-regulated cellular proteins including markers for proliferation, adherens junctions, and cytoskeleton organization using a combination of Western Blot analyses and immunocytochemistry techniques.
Task 6 progress in first year of funding:
1. Evidence for ability of parous matrix to induce a dormant state in tumor cells is evidenced by increased e-cadherin and beta-catenin junctional staining in cells plated onto parous matric in comparison to nulliparous matrix (**Fig 8a&b**). In this study, parous matrix "phenocopies" Matrigel, which is known to induce a quiescent/dormant state in D2.OR cells [5].
Proposed task 6, year two aims:
1. Quantitate E-cadherin junctional staining in 3D culture using the computer assisted Aperio system.
2. Expand IHC analysis to proliferation markers.
3. Validate quantitative IHC data by Western blot.

Task 7: We will evaluate if the „tumor suppressive" ECM signature can be identified in breast tissue of parous women and in women with anti-estrogen treatment compared to controls using immunohistochemical assays.
Months 12-24
a) Normal breast tissues from age matched nulliparous and parous women will be used to validate top candidates from the „tumor suppressive" ECM signature via IHC and quantitative histology. We have already collected biopsies from 20 women per group and fully regulatory compliance through our IRB has been secured.
b) Normal breast tissues from women treated four months in neoadjuvent setting with aromatase inhibitor exemestane alone or in combination with selective estrogen receptor modulator, tamoxifen will be used to validate top candidates from the „tumor suppressive" ECM signature via IHC and quantitative histology. These tissues will be obtained from the UCHSC Breast Center, the Denver Health Medical Oncology Breast Clinic, through Dr. Borges.
Task 7 progress in first year of funding: Not yet initiated.
Proposed task 7, year two aims:
1. Begin task of tissue collection from University of Colorado Tissue Bank.
2. Begin IHC staining and analyses.

List of Tables and Figures:
Table 1: Mammary gland ECM proteome data
Figure 1: Quantitative proteome data and protein interaction network.
Figure 2: Data demonstrating elevated fibrillar collagen with parity
Figure 3: In vivo collagen SHG analysis
Figure 4: In vivo xenograft models demonstrating host mediated suppression of cancer progression in parous mice.
Figure 5: Demonstration that isolated mammary matrices support mammary epithelial cell organization independent of parity status, as quality control assay for matrix**.**
Figure 6: Xenograft models demonstrating ECM mediated suppression of cancer progression in parous mice.
Figure 7: 3D culture showing suppression of ERK signaling and invasion by tumor cells on parous- matrix.
Figure 8: 3D culture showing gain of adherens junctions in tumor cell organoids on parous- matrix.

Table 1. Most Abundant ECM Proteins Identified in Rat Mammary Matrices:

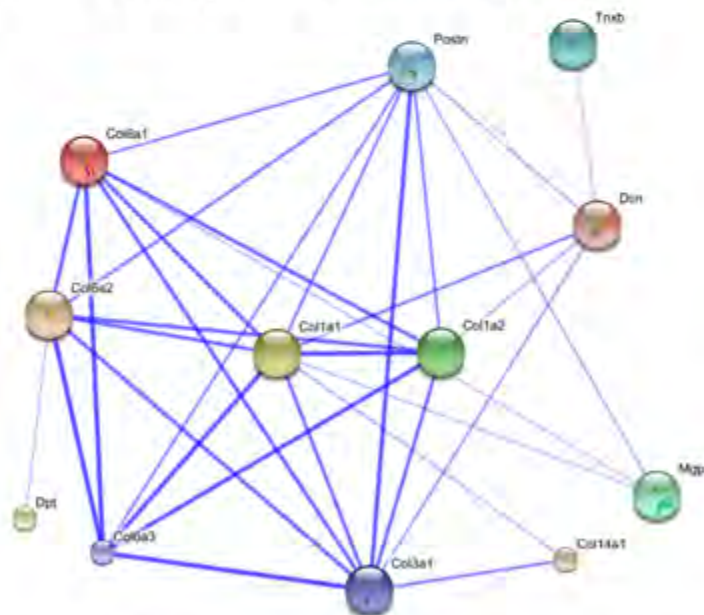| Accession	Description	Unique Peptides Used for Quantitation (N=5)		Sequence Coverage	
		Avg.	SD	Avg.	SD
IPI00188909	Col1a1 Collagen alpha-1(I) chain	179.7	10.5	67.3 (b1)	5.8
IPI00188921	Col1a2 Collagen alpha-2(I) chain	132.3	16.6	68.3 (b1)	4.0
IPI00565677	Col6a3 Collagen alpha-3(VI) chain	121.3	11.6	50.3 (b1)	8.1
IPI00366944	Col3a1 Collagen alpha-1(III) chain	111.7	6.1	57.3 (b1)	8.0
IPI00360766	Col14a1 Collagen alpha-1(XIV) chain	113.7	9.7	62.7 (b1)	5.0
IPI00951429	Fbn1 Fibrillin 1	99.0	14.5		
IPI00371853	Col6a1 Collagen alpha-1(VI) chain	45.3	6.4	48.0 (b1)	17.1
IPI00200757	Fn1 Isoform 1 of Fibronectin	28.7	15.4		
IPI00190088	Periostin	41.7	5.5		
IPI00372839	Col6a2 Collagen alpha-2(VI) chain	36.0	1.7	41.0 (b1)	11.8
IPI00199861	Dcn Decorin	27.0	1.0	59.3 (b8)	8.4
IPI00911228	Tnxb Tenascin X	25.0	2.0		
IPI00190287	Prolargin	24.3	0.6		
IPI00206403	Lum Lumican	18.0	2.0		
IPI00191090	Bgn Biglycan	17.7	0.6		
IPI00362931	Ogn Osteoglycin	17.7	2.1		
IPI00371512	Dpt Dpt protein	12.0	1.0		
IPI00214859	Col18a1 Collagen alpha-1(XVIII) chain	12.3	1.5		
IPI00326179	Fbln5 Fibulin-5	6.7	0.6		
IPI00201608	Col5a1 Collagen alpha-1(V) chain	3.3	1.5		
IPI00366945	Col5a2 Collagen alpha-2(V) chain	2.0	1.7		
IPI00211401	Mgp Matrix Gla protein	2.0	0.0		

Table 1. Mammary ECM proteins identified via mass spectrometry-based proteomics.
A list of identified ECM proteins from nulliparous and parous mammary glands. The list includes number of unique peptides used for quantitative analysis and percentage coverage for selected ECM proteins.

1A.
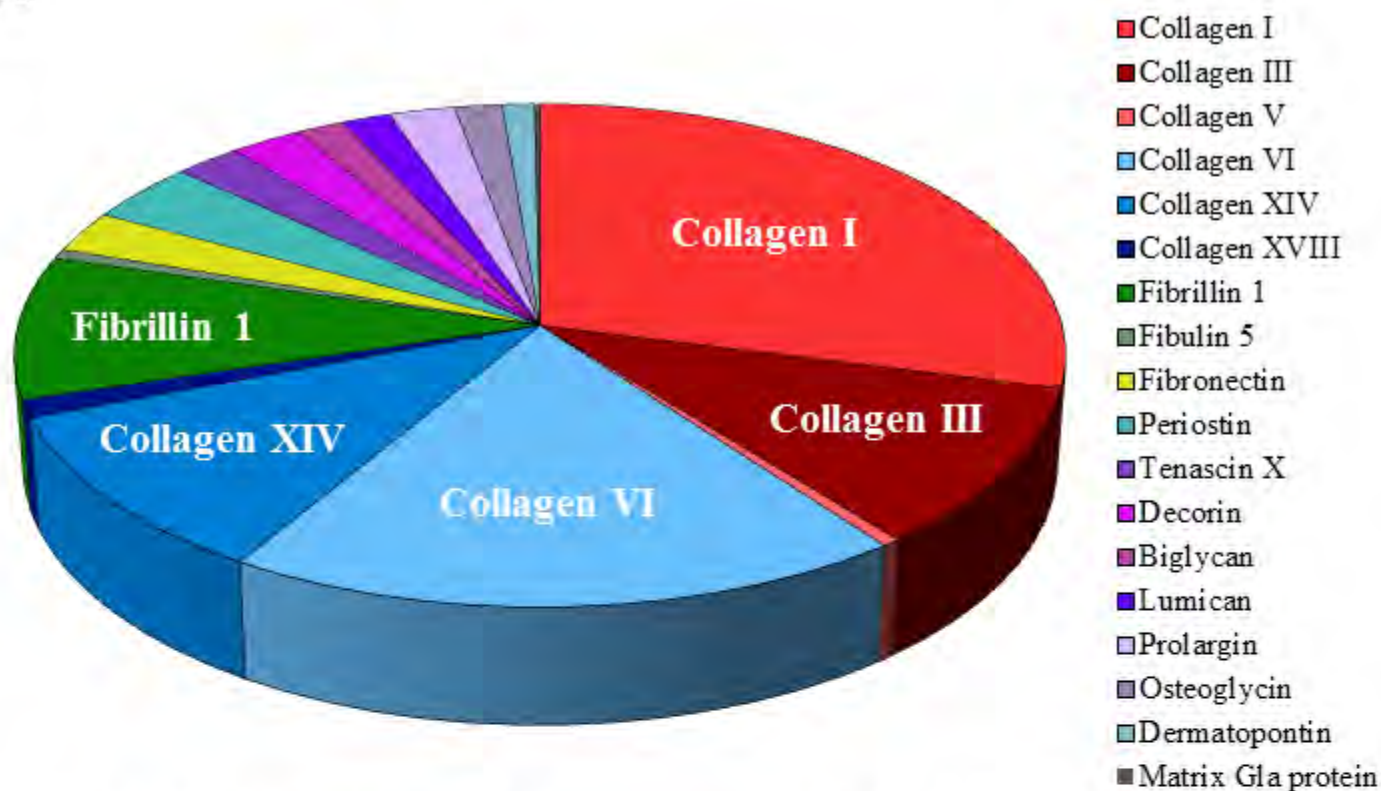
1B.   Protein-protein interactions in Mouse   Protein-protein interactions in Human

Figure 1. Mammary ECM proteins identified via mass spectrometry-based proteomics.
(A) A pie chart based on the number of unique peptides for ECM proteins described in Table 1. (B) Confidence view for protein-protein interactions in mouse and human using STRING 9.0. Thicker lines represents stronger association among proteins.
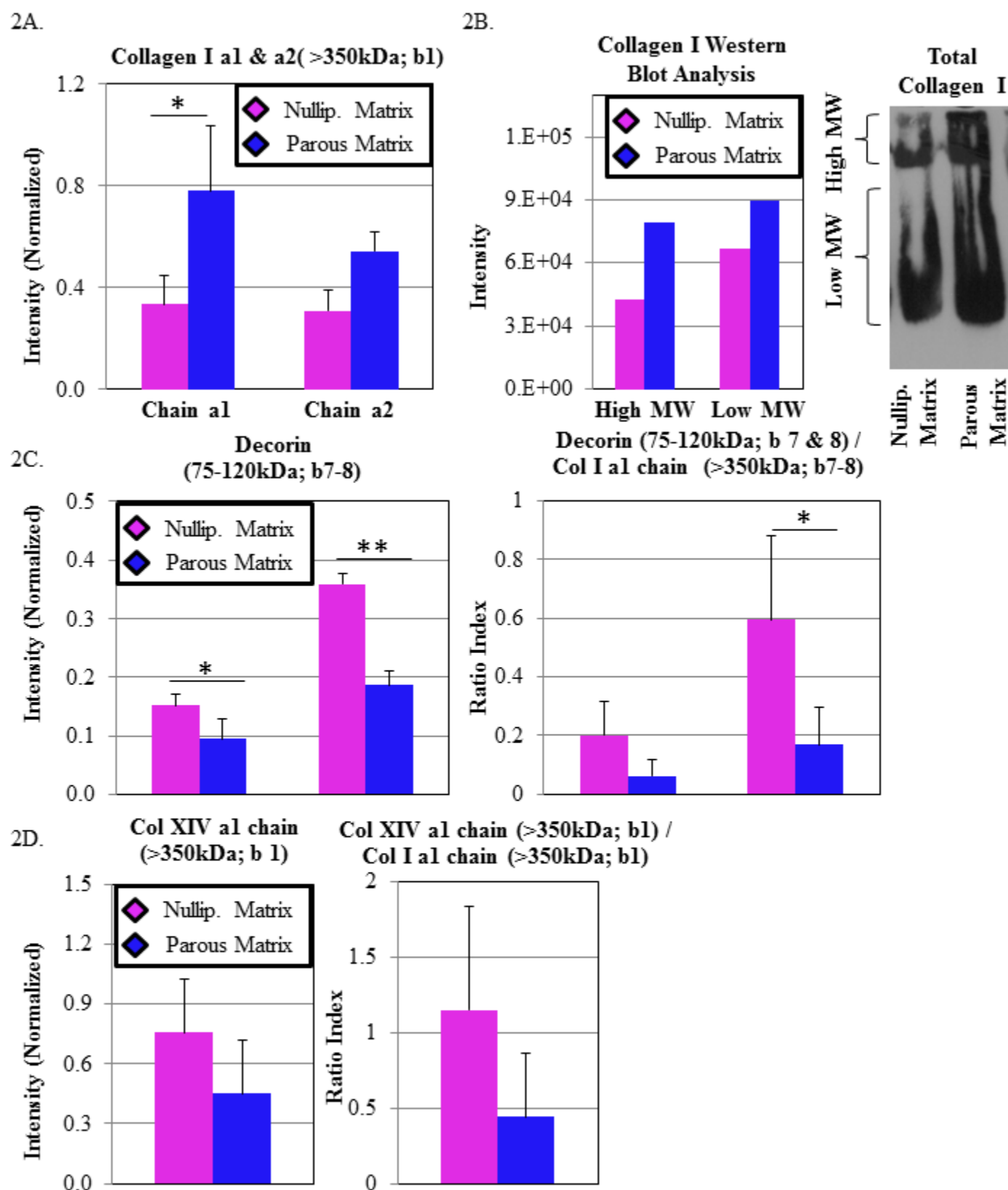
Figure 2. Parity-related compositional differences between nulliparous and parous mammary ECM. (A) Collagen I α1 and α2 chains abundance in nulliparous or parous mammary ECM via mass spectrometry-based proteomics and label-free quantitative analysis. Means are calculated from an N=5; * p<0.01, ANOVA. (B) Total Collagen I levels evaluated via WB. (C) Decorin and (D) collagen XIV α1 chain abundances are evaluated as described in (A) and ratios for decorin or collagen XIV to collagen I α1chain are shown. *P<0.05 **P<0.001 ANOVA.

8
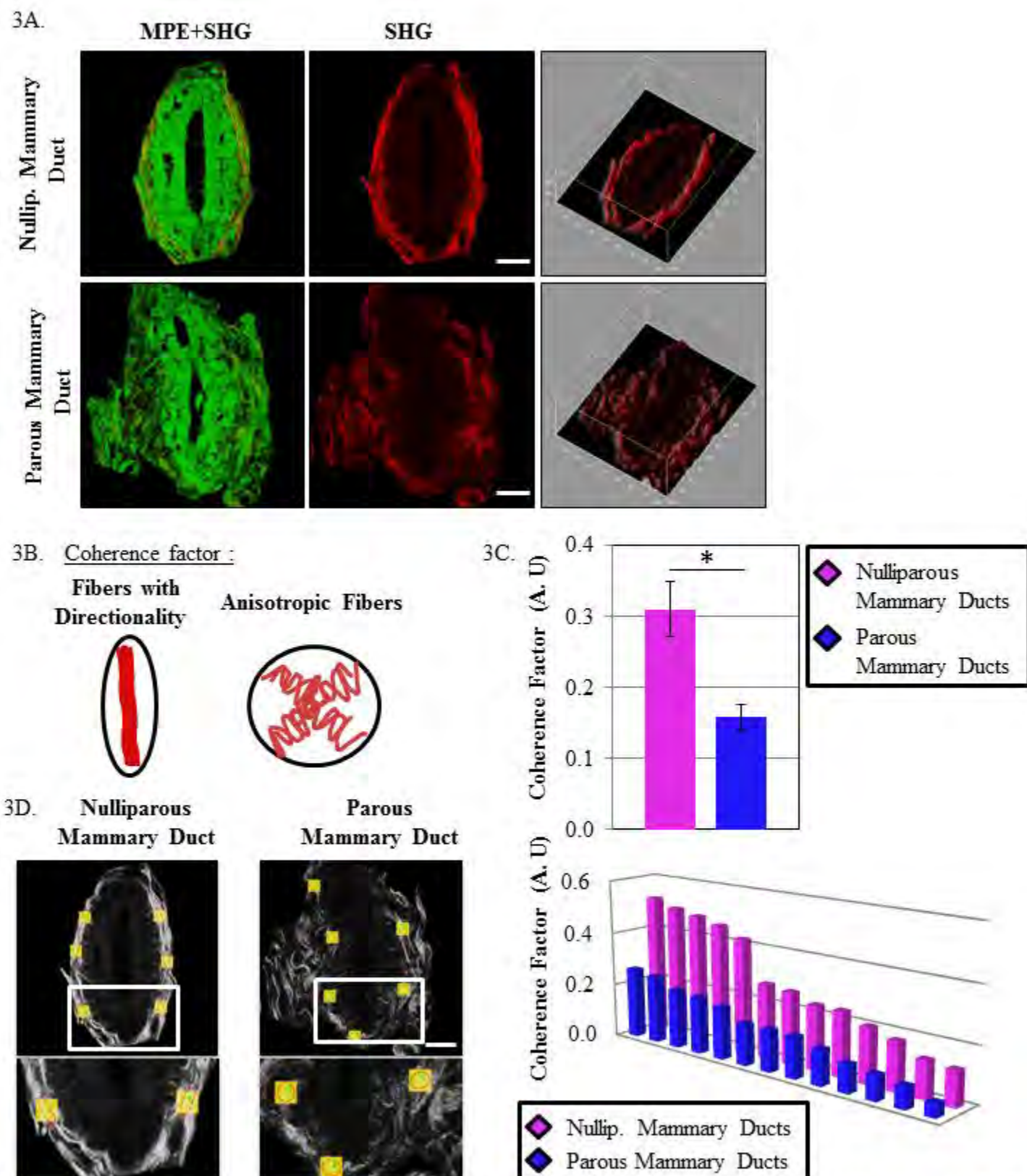
Figure 3. Fibrillar collagen adjacent to mammary ducts from parous rats is more randomly organized. (A) Mammary ducts imaged using multiphoton excitation (MPE) to generate intrinsic signals for cellular and stromal autofluorescence and second harmonic generation (SHG) signal to detect fibrillar collagen. (B) A diagram to illustrate the various degrees of fiber directionality. (C) and (D) Collagen fiber directionality (coherence factor) quantitated via ImageJ. N=13 size-matched mammary ducts of 7 nulliparous or parous female rats. * p <0.002 Unpaired t-test. Scale bars represent 15 μm.

4A.
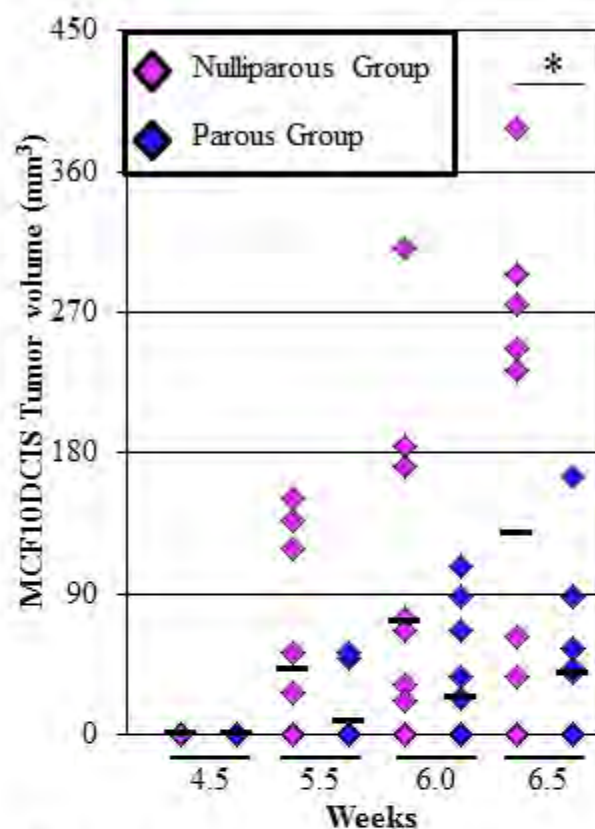
4B.
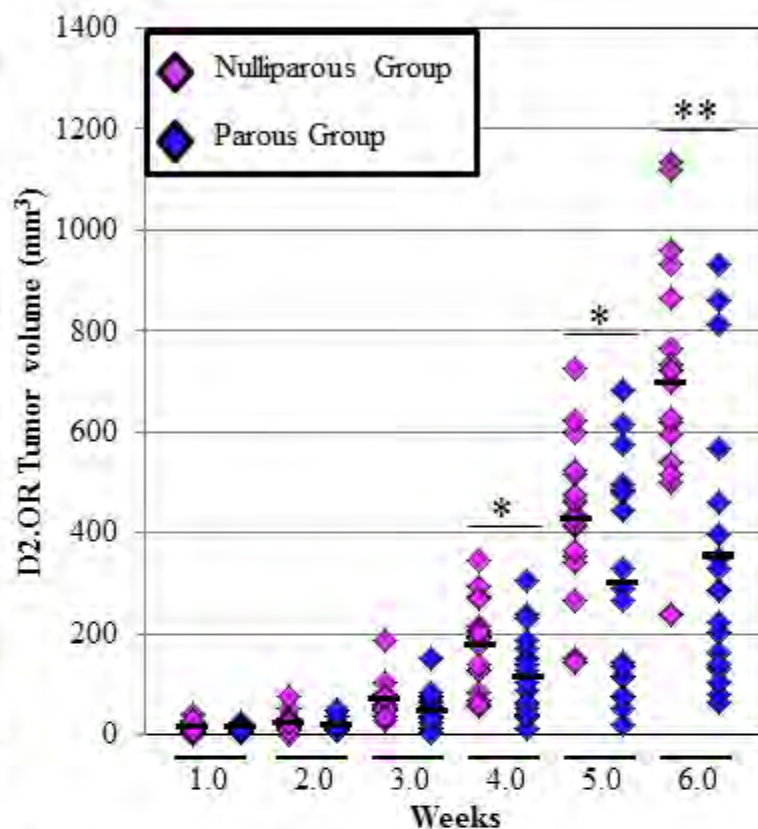
Figure 4. Parous mammary environment reduces tumor growth *in vivo*. Human breast cancer MCF10DCIS cells (A) and murine mammary tumor D2.OR cells (B) injected into the mammary fat pads of nulliparous and parous SCID mice. * p<0.025, **p<0.0005, Unpaired t-test. (C) MCF10DCIS cells co-injected with nulliparous or parous mammary ECM into mammary fat pad of nulliparous SCID mice. * p value < 0.01, Unpaired t-test.

Figure 5. Human normal mammary epithelial MCF12A cells form complex and healthy 3D structures in nulliparous or parous mammary ECM. Representative brightfield images of MCF12A in 3D culture at day 4. Scale bar represents 100μm.

Figure 6. Parous mammary ECM co-injected with tumor cells results in a decreased tumor growth. MCF10DCIS cells co-injected with nulliparous or parous mammary ECM into mammary fat pad of nulliparous SCID mice. * p value < 0.01, Unpaired t-test.

7A.

H&E

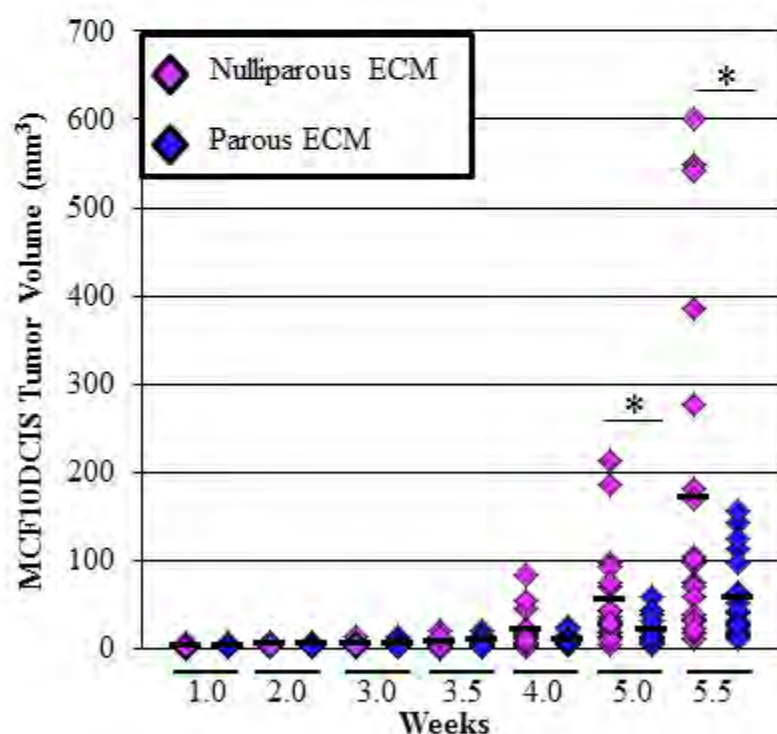D2.OR cells in Nullip. Matrix

D2.OR cells in Parous Matrix

Scale bar represents 50μm

7B.

Phospho/Total ERK Ratio

□ ERK 1
■ ERK 2

Nullip. Matrix   Parous Matrix

D2.OR Cell Lysates from 3D Culture
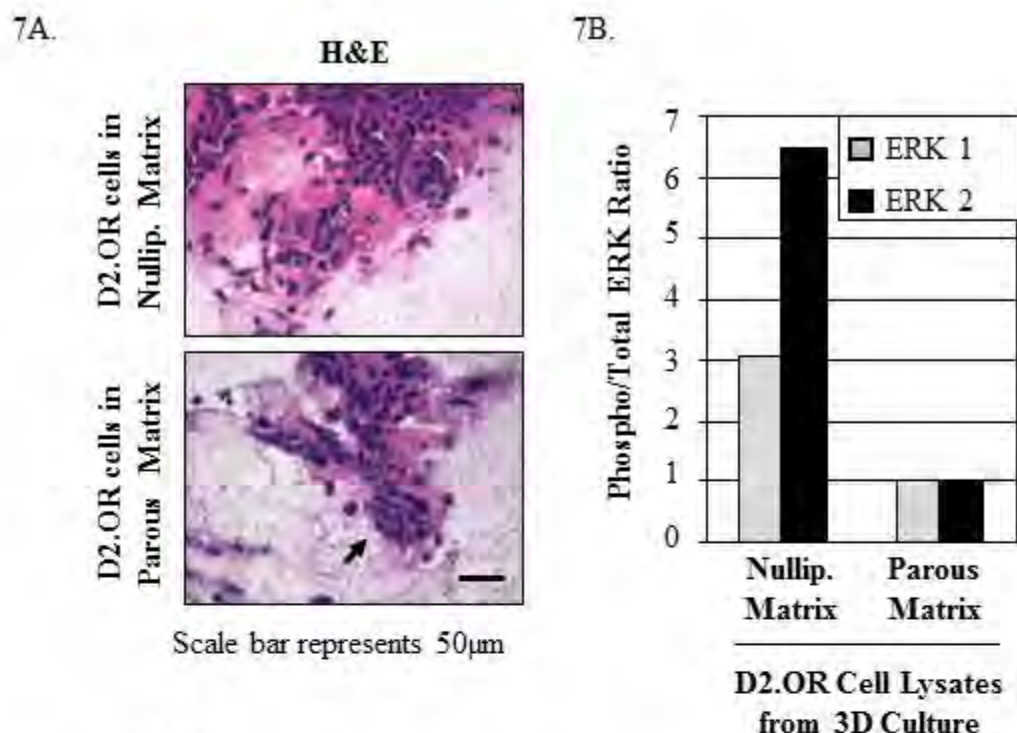
Figure 7. Parous mammary ECM suppresses invasive morphology of tumor cells *in vitro*. (A) Representative H&E images of murine mammary tumor D2.OR cells in the presence of nulliparous or parous mammary ECM in 3D culture at day 4. Scale bar represents 50μm. (B) Evaluation of ERK phosphorylation in D2.OR cells lysates via Western Blot.

8A.

D2.OR cells in Matrigel   D2.OR cells in Nullip. Matrix   D2.OR cells in Parous Matrix

E-Cadherin

β-catenin

Scale bar represents 20μm

8B.

Junctional β-Catenin/ # of Total Cells

*

Matrigel Nullip. Parous
          Matrix Matrix

Figure 8. Parous mammary ECM better supports adherens junctions. (A) representative images of E-cadherin and total β-catenin staining of D2.OR cells in the presence of nulliparous or parous mammary ECM in 3D Culture at day 4. Scale bar represents 20μm. (B) Quantitative analysis for junctional β-catenin staining shown in (A). * p value < 0.05, ANOVA.

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KEY RESEARCH ACCOMPLISHMENTS:
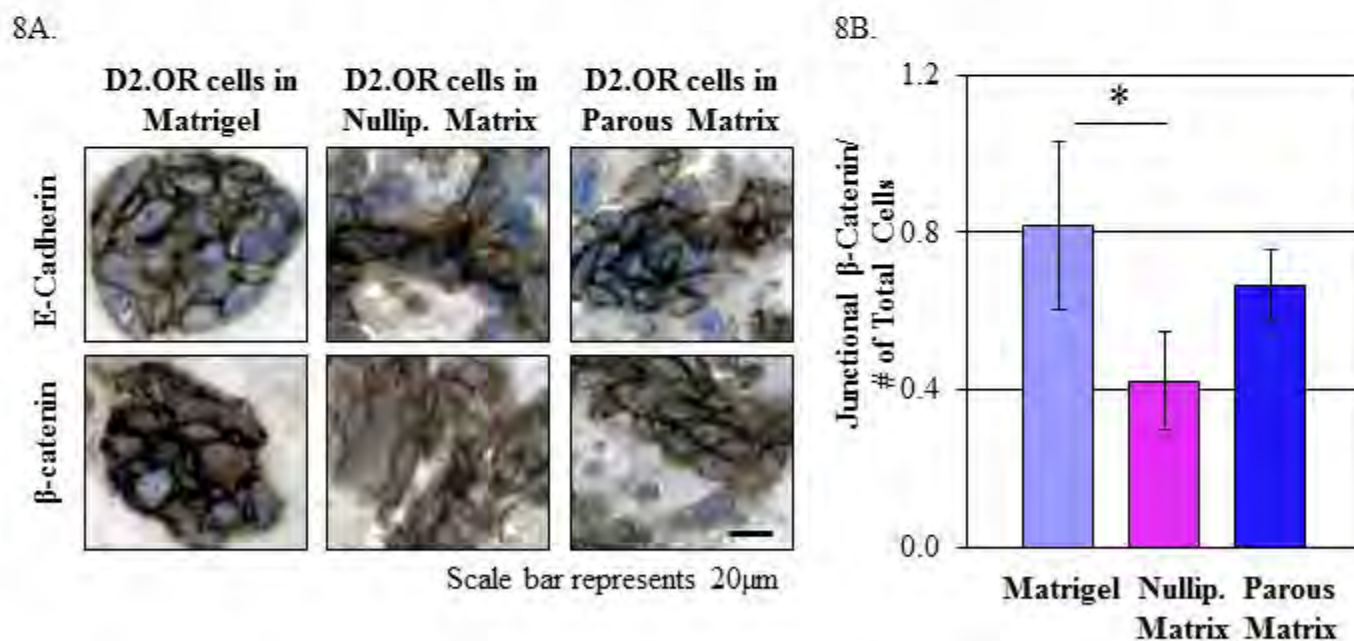1. Performed first qualitative ECM proteomic analysis of mammary ECM proteins.
2. Using isolated mammary ECM preparations as substratum for breast cancer cells, we have identified mammary ECM as mediating some of the protective effect of parity on breast cancer.
3. Characterized the parous mammary gland as having elevated levels of fibrillar collagen, an observation that is opposite of expectations. Higher levels of fibrillar collagen have been shown to be tumor promotional in numerous *in vitro* and *in vivo* studies [2]. Further, increased breast density is a dominant risk factor in women, and part of mammographic density is due to fibrillar collagen content.
4. Addressed the paradox of high fibrillar collagen associating with cancer cell suppression by evaluating collagen structure. We performed second harmonic generated imaging of collagen and found that the organization of collagen is distinctly different between nulliparous and parous mammary glands.
5. Identified collagen of the parous gland as being less cohesive and more anisotropic, consistent with the collagen being less stiff and more compliant.
6. These results provide insight into changes that occur in the mammary microenvironment due to reproductive history, and may impact tumor cell dormancy.
7. These results could have implications for interpreting breast density in women, as high breast density may fall into discrete subclasses defined by organization of the collagen and not just abundance.

REPORTABLE OUTCOMES:

National Presentations: Pepper Schedin, Mammary extracellular matrix proteins contribute to the protective effect of parity and may provide insight into tumor cell dormancy, Symposium 11, Silence is Golden: Key Issues in Dormancy and Recurrence, DOD Era of Hope, Orlando, Florida, August 3, 2011.

Manuscripts:
1. Maller O, Martinson H, and **Schedin P.** Extracellular matrix composition reveals complex and dynamic stromal-epithelial interactions in the mammary gland. J of Mammary Gland Biology and Neoplasia 2010; 15(3): 301-318. PMID: 20811805

Abstracts:
1. *Mammary extracellular matrix proteins contribute to the 'protective effect' of parity and may provide insight into tumor cell dormancy*, **Pepper Schedin**, Ori Maller, Traci Lyons, and Kirk Hansen, Poster presentation, DOD Era of Hope, Orlando, Florida, August 3, 2011.
2. *Mammary extracellular matrix proteins contribute to the 'protective effect' of parity and may provide insight into tumor cell dormancy*. **Ori Maller**, Kirk Hansen, Traci Lyons, Pepper Schedin, Poster presentation, Gordon Research Conference on Mammary Gland Biology, Newport, RI. June 7-12, 2011.
3. *The function of extracellular matrix in breast cancer dormancy.* **Ori Maller**, Traci Lyons, Jeffrey Green, Pepper Schedin, Joint Metastasis Research Society-AACR Conference on Metastasis and Tumor Microenvironment- Dormancy Session, Poster presentation, Philadelphia, PA. September 2010.

Awards:
AACR Scholar-in-Training Award to Ori Maller – Special Conference on Metastasis and Tumor Microenvironment, 2010.

Employment or research opportunities:
1. Pre-doctoral training for Ori Maller, whose thesis work is fully funded by this award.
2. Undergraduate student training for Nicole Snelgrove, Metropolitan State College Work Study student, Denver Colorado.

Informatic data bases: none at present, but mammary gland ECM proteome will be publically available at time of publication.

Funding applied for based on this work: none at present.

CONCLUSIONS: Our studies thus far have uncovered that randomly organized fibrillar collagen contributes to the "protective effect" of parity against breast cancer. Understanding how collagen organization and levels of collagen associated proteins are altered between "tumor supportive" and "tumor suppressive" microenvironments could have array of implications for studying breast cancer progression, response to chemotherapy and dormancy in both primary and metastatic sites.

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1.   Maller, O., H. Martinson, and P. Schedin, *Extracellular matrix composition reveals complex and dynamic stromal-epithelial interactions in the mammary gland.* J Mammary Gland Biol Neoplasia, 2010. **15**(3): p. 301-18.
2.   Schedin, P. and P.J. Keely, *Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression.* Cold Spring Harb Perspect Biol, 2011. **3**(1): p. a003228.
3.   Conklin, M.W., et al., *Aligned collagen is a prognostic signature for survival in human breast carcinoma.* Am J Pathol, 2011. **178**(3): p. 1221-32.
4.   Abrams, T.J., et al., *Changes in the parous rat mammary gland environment are involved in parity-associated protection against mammary carcinogenesis.* Anticancer Res, 1998. **18**(6A): p. 4115-21.
5.   Barkan, D., et al., *Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment.* Cancer Res, 2010. **70**(14): p. 5706-16.

APPENDICES: MANUSCRIPS, ABSTRACTS

Extracellular Matrix Composition Reveals Complex and Dynamic Stromal-Epithelial Interactions in the Mammary Gland

Ori Maller · Holly Martinson · Pepper Schedin

Abstract The mammary gland is an excellent model system to study the interplay between stroma and epithelial cells because of the gland's unique postnatal development and its distinct functional states. This review focuses on the contribution of the extracellular matrix (ECM) to stromal-epithelial interactions in the mammary gland. We describe how ECM physical properties, protein composition, and proteolytic state impact mammary gland architecture as well as provide instructive cues that influence the function of mammary epithelial cells during pubertal gland development and throughout adulthood. Further, based on recent proteomic analyses of mammary ECM, we describe known mammary ECM proteins and their potential functions, as well as describe several ECM proteins not previously recognized in this organ. ECM proteins are discussed in the context of the morphologically-distinct stromal subcompartments: the basal lamina, the intra- and interlobular stroma, and the fibrous connective tissue. Future studies aimed at in-depth qualitative and quantitative characterization of mammary ECM within these various subcompartments is required to better elucidate the function of ECM in normal as well as in pathological breast tissue.

Keywords Extracellular matrix · Stromal-epithelial interactions · Basal lamina · Collagen · Fibronectin · Laminin

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Abbreviations

collagen IV	type IV collagen
DDR1	discoidin domain receptor 1
EM	electron microscopy
EGFR	epidermal growth factor receptor
ECM	extracellular matrix
ED	extra domain
FACIT	fibril associated collagens with interrupted triple helices
FGF	fibroblast growth factor
FN	fibronectin
GAG	glycosaminoglycan
H&E	hematoxylin and eosin stain
HGF	hepatocyte growth factor
LN	laminin
LAP	latency-associated peptide
LRR	leucine-rich repeats
LOX	lysyl oxidase
MMP	matrix metalloproteinase
MAGP	microfibril-associated glycoprotein
MCP-1	monocyte chemoattractant protein-1
SPARC	secreted protein acidic and rich in cysteine
SLRP	small leucine-rich proteoglycan

SD	Sprague Dawley
TN	tenascin
tTG2	tissue transglutaminase 2
TGF-β	transforming growth factor β
TLR	toll like receptor

Introduction

The mammary gland is an excellent model system to study the function of stromal-epithelial interactions because of the gland's unique development across its several distinct functional states, depending on reproductive state. Unlike most organs, which develop to morphological maturity during embryogenesis, the majority of mammary ductal morphogenesis occurs with the onset of ovarian function. Further, with each estrous (rodent) or menstrual (human) cycle, the alveoli undergo cyclic expansion and maturation, followed by a modest regression phase as ovarian hormone levels rise and fall, respectively [1–4]. The mammary gland is also unique in that terminal differentiation does not occur unless pregnancy and lactation ensue [5]. These unique attributes highlight the dynamic, non-homeostatic nature of the normal mammary gland.

Since mammary gland development is largely postnatal, organogenesis and differentiation are extended in time, occurring over a period of weeks in the rodent, rather than hours and days as observed in other organs. Further, in rodents, the development of mammary glands occurs along the entire flank of the animal with 5 pairs of glands in the mouse and 6 pairs in the rat. As a result, there is sufficient mammary tissue in these species to permit physical, functional, and biochemical characterizations. These features make the rodent mammary gland a highly suitable model to evaluate the interplay between stroma and epithelium throughout gland development and during distinct functional states such as pregnancy or lactation.

The focus of this review is on the extracellular matrix (ECM) component of the mammary stroma. ECM can be described as an interconnected meshwork of secreted proteins that interacts with cells to form a functional unit [6]. Cell-ECM interactions have been implicated in cell adhesion, survival, apoptosis, polarity, proliferation, and differentiation. ECM confers these cellular functions through structural properties such as physical support and boundary constraints, classical signal transduction pathways, and by providing mechanosensory cues [7]. Epithelial cells receive instructive cues from the ECM via integrin receptors and the non-integrin receptors such as discoidin domain receptor 1 (DDR1) [8], dystroglycan [9], and syndecan [10]. Integrins are transmembrane receptors composed of α and β subunits, with 18 α and 8 β subunits that

heterodimerize to generate 24 canonical integrin receptors [11, 12]. A comprehensive description of ECM-integrin interactions is beyond the scope of this review; the reader is referred to reviews by Desgrosellier et al. and Larsen et al. [13, 14].

ECM synthesis and assembly in the mammary gland are the product of many cell types including epithelial cells, myoepithelial cells, fibroblasts, adipocytes, endothelial cells, and immune cells [15–17]. Bissell and colleagues describe the relationship between ECM and mammary epithelial cells as dynamic and reciprocal, leading to two-way communication, where ECM instructs and supports cells, while cells build, shape, and re-shape the ECM [18]. Mammary ECM can be separated into three broad structural categories: the highly-specialized basal lamina that directly abuts the basal side of mammary epithelial cells (Fig. 1), the intra- and interlobular stroma immediately adjacent to alveoli and lobules, respectively (Figs. 1 and 2), and the fibrous connective tissue that is devoid of epithelium (Fig. 2). In this review we explore the composition and function of each of these ECM categories using data obtained largely from rodent models. Although rodent models recapitulate many aspects of human mammary gland biology, there are interspecies variations in mammary stroma composition, organization, density, and function. Thus, while significant insight into mammary ECM is obtained through the study of rodents, future studies validating results in human breast tissue are essential.

Rodent Mammary Gland as a Model System to Study Human Breast

The mouse and rat models recapitulate critical aspects of human mammary gland development and function. The rodent mammary gland and human breast have similar rudimentary mammary parenchyma, which consist of a layer of luminal epithelial cells, a basal epithelial cell layer that putatively contains mammary stem cells, and the outer contractile myoepithelial cells adjacent to the basal lamina ECM [19]. The parenchyma structure consists of a single elongated ductal tree (rodent) or multiple ductal networks (human) that develop by bifurcation of specialized end bud structures present during puberty [20]. While the overall epithelial structure is similar between humans, rats, and mice, the relative abundance of connective tissue varies considerably. Stroma surrounding the lobules and ducts (intra- and interlobular stroma) in mice is sparse and there is little acellular fibrous connective tissue between ducts. Instead, the mammary glands of mice are characterized by an abundance of white adipose tissue that is directly adjacent to the sparse interlobular stroma (Fig. 2c). In
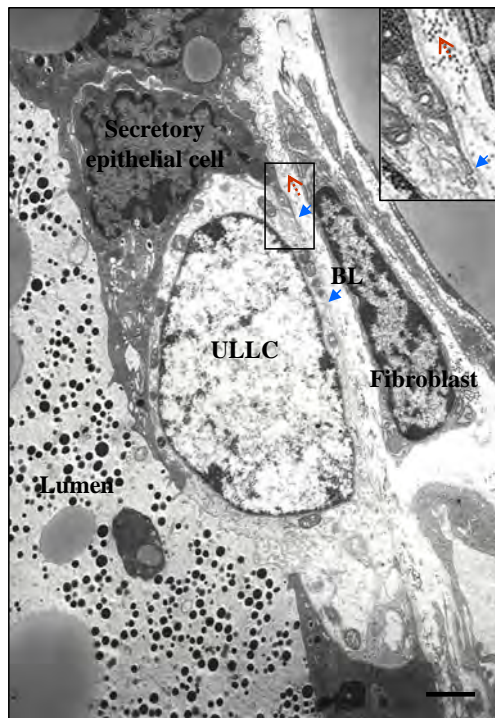
Figure 1 Electron micrograph image of mammary epithelial cells from a lactating mouse. The solid blue arrow points to the basal lamina (BL) adjacent to a putative mammary progenitor cell known as undifferentiated large light cell (ULLC), and a secretory epithelial cell. The dashed red arrow points to intralobular fibrillar collagen adjacent to a fibroblast. Scale bar represents 5 microns

humans the ratio of fibrous connective tissue to adipose tissue is opposite, with an abundance of stroma surrounding the alveoli and ducts, a predominance of fibrous connective tissue between ducts, and reduced adipose content (Fig. 2a). Organization of the stroma in outbred Sprague Dawley (SD) female rats is intermediate between mice and humans and thus is histologically more similar to humans than is the mouse (Fig. 2b) [21]. While the mechanisms driving the different ratios of fibrous connective to adipose tissues across species are currently unknown, we suggest a relationship to ovarian hormone exposure, particularly progesterone, may exist. Compared to mice, the SD rat has a robust luteal phase during the estrus cycle, resulting in cyclical progesterone exposure that is more similar to that for women [1]. Given that pituitary and ovarian hormone exposure drives most aspects of gland development and adult function, instructive cues from hormones are expected to also directly or indirectly influence composition and organization of the adjacent stroma [22–24]. Hence, studying the hormonal regulation of normal mammary gland biology may afford novel insights into how stromal organization is regulated under physiological conditions, which can be utilized to decipher this relationship in terms of tissue plasticity and breast disease. However, systemic/

host exposures are unlikely to completely explain variation in stromal density given that very distinct microenvironments can exist within individual adjacent lobules (Fig. 3).

Pleiotropic Roles for ECM in the Mammary Gland

The ECM provides physical support that is essential for overall tissue architecture. Changes in ECM properties are communicated to epithelial cells via transmembrane receptors that provide a conduit between the ECM, the cytoskeleton and its transcriptional machinery [25]. Thus, ECM interactions ultimately determine epithelial cell phenotype. A role for ECM stiffness in the mammary gland has been increasingly explored including the role of stiffness requirements for establishing apical/basal cell polarity and lateral cell junctions [26], as well as for cell proliferation [27, 28]. Importantly, Discher and colleagues have determined that matrix stiffness and mechanotransduction are seminal factors in mesenchymal stem cell lineage specification [29]. By modulating matrix stiffness in vitro, this group demonstrated that mesenchymal stem cells acquired a neuronal gene profile on soft matrix, while acquiring osteoblast-like properties on rigid matrix [29]. In the mammary gland, $\beta1$ integrin is a cellular "mechanosensor" that is imperative for maintaining basal progenitor cells, as demonstrated by inability of mammary epithelium lacking $\beta1$ integrin to repopulate the gland upon serial transplantation [30]. These data suggest that physical properties and site-specific composition of adjacent basal lamina ECM may contribute to the "stemness" of mammary progenitor cells through $\beta1$ integrin.

In order to understand how ECM stiffness influences the function and fate of mammary epithelial cells, it is vital to understand its composition as well as its architecture, which is largely defined by protein-protein interactions. While secreted ECM proteins can interact through numerous, weak non-covalent bonds, crosslinking these proteins via covalent chemical bonds is essential for ECM scaffold organization, tensional characteristics, and stabilization. Lysyl oxidase (LOX), a copper-dependent enzyme, catalyzes intra- and intermolecular crosslinking of fibrillar collagens and elastic fibers through oxidative deamination of lysine residues [28, 31, 32]. LOX activity has been shown to promote tissue stiffness and fibrosis as well as modulate elastic properties of elastic fibers, establishing LOX as a key regulator of tissue tension [28, 31–33]. Tissue transglutaminase 2 (tTG2) has also been implicated in crosslinking ECM proteins in the mammary stroma. The crosslinking activity of tTG2 depends on calcium and proceeds by catalyzing covalent bonds between glutamine and ϵ-amino groups of lysine residues [34]. Future studies are needed to examine how LOX and tTG2 are regulated in

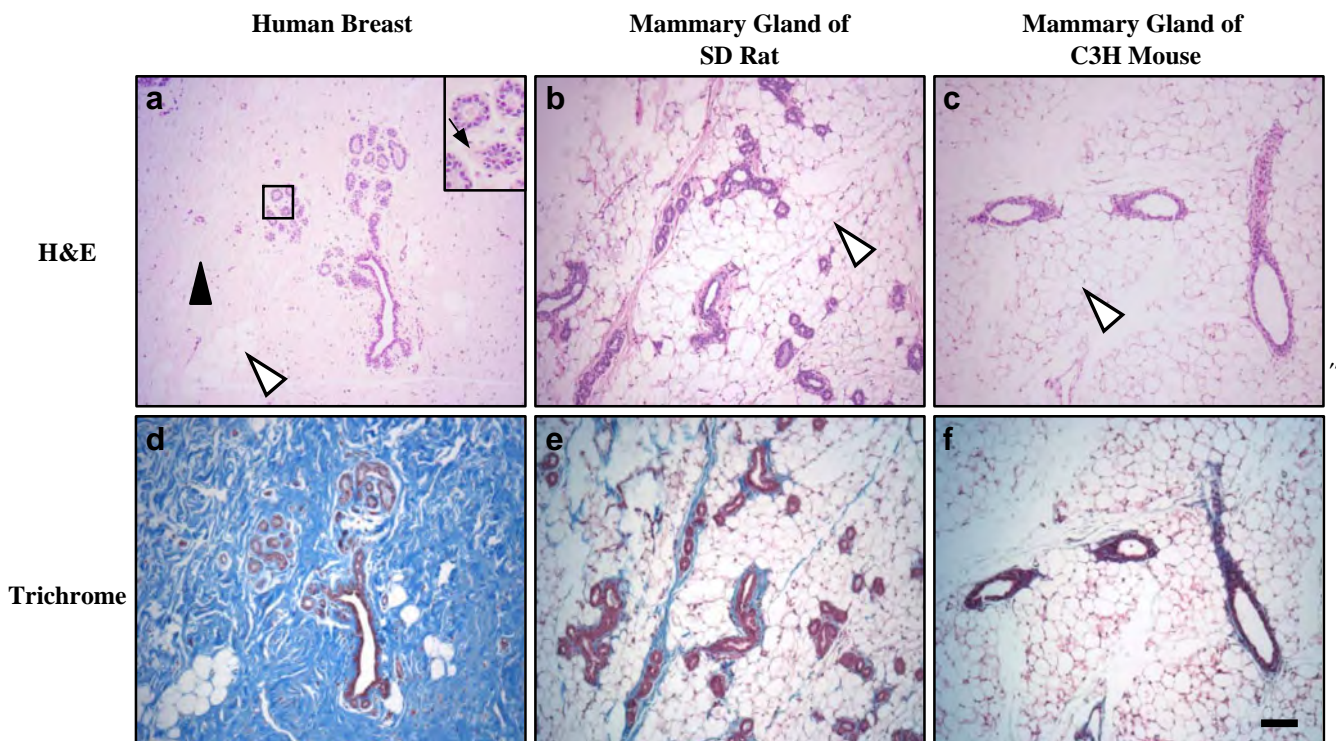| Human Breast | Mammary Gland of SD Rat | Mammary Gland of C3H Mouse |

Figure 2 Histological sections from human breast and rodent mammary gland from nulliparous females. **a–c** H&E-stained nulliparous human breast tissue (**a**), nulliparous SD rat mammary tissue (**b**), and nulliparous C3H mouse mammary tissue (**c**). In **a**, solid arrow head points to fibrous connective tissue (pink) that is dominant in human breast tissue. Arrow in the inset box points to intralobular stroma. Open arrow heads point to adipocytes, which are abundant in rat and mouse glands. **d–f** Serial sections stained with Masson's trichrome showing collagen as blue fibers in human nulliparous breast tissue (**d**), nulliparous SD rat mammary tissue (**e**), and nulliparous C3H mouse mammary tissue (**f**). Scale bar represents 100 microns

the mammary gland and to determine their unique contributions to ECM function.

ECM as a Reservoir and a Source for Growth Factors and Cytokines

Growth factors and cytokines can bind to ECM proteins, identifying another avenue by which ECM can regulate mammary morphogenesis and function. For example, transgluaminase crosslinks the large latent complex of transforming growth factor β (TGF-β), known as latent TGF-β binding protein, to ECM proteins [35]. Growth factors and cytokines bind to ECM through glycosaminoglycans (GAG), which are anionic linear polysaccharides consisting of repeating hexuronic acid and hexosamine. GAG can be thought of as "glue" that allows growth factors to "stick" to basal lamina and fibrillar ECM proteins. Interestingly, different classes of GAGs are associated with distinct aspects of mammary ductal elongation [20]. Silberstein et al. and Williams et al. used electron microscopy (EM) to show that the basal lamina surrounding the tips of end buds in mice is thin and rich in hyaluronic acid, while the basal lamina along the flank of end buds is thicker and associated with sulfated

GAGs [20, 36]. These observations are interesting because the terminal end bud tips are actively elongating structures while the flank of the end bud is not [37]. These observations raise questions on how GAG composition contributes to ECM thickness, rigidity, and the ability of ECM to serve as a reservoir for growth factors and cytokines, as well as the ability of epithelial cells to penetrate into the surrounding stroma and proliferate.

Release and activation of growth factors and cytokines from ECM can occur by altering matrix stiffness and/or inducing ECM proteolysis [38, 39]. For example, TGF-β activity can depend on fibrillar ECM proteolysis or altered matrix stiffness. TGF-β is secreted as a large latent complex (LLC) by multiple cell types and associates with fibrillar ECM proteins [40]. TGF-β is activated by proteolytic cleavage of the latency-associated peptide (LAP) or by conformational change in LAP through binding to the αvβ6 integrin receptor [41, 42]. However, Hinz and colleagues recently published evidence suggesting that mechanical tension is also involved in the release of TGF-β1 from the ECM [43]. They suggested that high contractile activity of myofibroblasts on a stiff matrix may lead to a conformational change in LAP, resulting in TGF-β1 release, while on soft matrix the latent complex stays

Human Breast

H&E

Figure 3 H&E-stained nulliparous human breast tissue. This histological section demonstrates how adjacent lobules can exist in very distinct microenvironments. The arrow points to fibrous intralobular stroma and the arrow head points to adipocyte-rich stroma. Scale bar represents 100 microns

intact due to lack of mechanical stretch [43]. Additional support for mechanical tension regulating ECM function is presented in studies demonstrating how fibroblast traction forces lead to large-scale directional patterning of fibrillar ECM proteins such as collagen I [44, 45]. Given that ECM tensional requirements and organization are expected to change throughout mammary gland development, it is likely that the ability of ECM to serve as a reservoir for growth factors and cytokines would be altered accordingly.

During expansion of the ductal network, Daniel and colleagues postulated that TGF-β1 regulates branching morphogenesis by inhibiting ductal growth through direct suppression of epithelial cell proliferation and by stimulating production of collagen I, GAG, and chondroitin sulfate within the periductal stroma at puberty [46]. Evidence for this proposal was obtained by administering TGF-β1 directly into the mammary gland of young female mice, which resulted in premature inhibition of ductal elongation concurrent with aberrant and increased deposition of ECM proteins [46, 47]. Moreover, as discussed above, these researchers observed high expression of TGF-β3 in the flank region of end buds, suggesting it may positively regulate ECM production at this site [48]. Given the growth suppressive role of TGF-β during pubertal gland development, it was surprising that expression of TGF-β2 and TGF-β3 transcripts was elevated during pregnancy [48]. As mentioned by Robinson et al. [48], this observation is not consistent with the growth inhibitory function that TGF-β(s) has on mammary epithelial cells during ductal elongation. A later study suggested that TGF-β1 only impedes epithelial cell growth in ducts, but not in alveoli, although the mechanism of this differential effect is unknown [49]. During lactation, the expression of TGF-β(s) is significantly downregulated [48], which may prevent TGF-β(s) from negatively regulating expression of milk proteins such as β-casein [48, 50, 51]. Upon the onset of involution when the gland remodels toward its prepregnant state, there is an upregulation of TGF-β transcripts, particularly for TGF-β3 [52]. TGF-β signaling may further contribute to the remodeling of the involuting gland by inducing ECM production, upregulating MMP expression, and by recruiting immune cells [53–55]. Taken together, TGF-β(s) expression and activation are tightly regulated in mammary gland development where they mediate stromal-epithelial interactions, in part by orchestrating ECM deposition and remodeling.

Instructive cues are also provided to cells from the ECM by the controlled release of protease-cleaved ECM fragments, termed 'matricryptins' [56, 57]. These fragments exhibit altered biological functions compared to intact ECM proteins. For example, fibronectin (FN) fragments initiate apoptosis in epithelial cells [58], while intact FN promotes cell adhesion and proliferation [59, 60]. A subclass of matricryptins, "matrikines," are short peptides from ECM proteins that function in a similar manner as cytokines [56, 57]. Instructive cues from ECM fragments will be further discussed below in the context of immune cells and ECM remodeling.

ECM Composition in the Mammary Gland

In order to understand the myriad of functions mediated by the ECM, one must have an in-depth understanding of the ECM proteins that contribute to each of the distinct ECM subcompartments. Our current understanding of ECM composition is rudimentary and largely derives from studies of rodent mammary gland tissue (mouse and rat). Until recently, the standard method for identifying ECM proteins was by biochemical isolation and Edman sequencing and/or by antibody-based detection of candidate proteins. Mass spectrometry-based proteomic analysis provides an unbiased, high-throughput comprehensive overview of ECM composition. Unfortunately, the traditional digestion approaches required to prepare proteins for proteomic analysis have proven ineffective for ECM proteins due to their resistance to proteolysis. In the past few years, Liang and others have used media conditioned by mammary epithelial cells in vitro to analyze their secretomes and have identified peptides originating from ECM proteins such as FN, laminin (LN), and type IV collagen (collagen IV) [61]. An in-solution digestion approach for trypsin-resistant ECM proteins was recently developed by Hansen et al., permitting the identification of known and previously unknown ECM proteins within the rat mammary gland [62]. In the next section of this review, we describe many of the well-known ECM proteins in the

mammary gland and highlight several ECM proteins not previously recognized. We discuss these ECM proteins in the context of the three distinct subcompartments depicted in Figs. 1 and 2.

Basal Lamina

The terms basal lamina and basement membrane have been used interchangeably to refer to the highly specialized ECM directly underlying the epithelium. While basement membrane refers to the ECM adjacent to the epithelium that is visible by light microscopy, the basal lamina is a sub-light microscope structure visualized only by EM. The basal lamina lies immediately beneath the epithelium and is a highly organized 20–100 nm thick structure (Fig. 1). Basal lamina in the mammary gland is thicker along the flanks of the terminal end bud compared to the growing bud tip, which correlates with compositional and possibly functional differences, as mentioned above [36]. The basal lamina consists of three layers: the middle electron dense layer termed the lamina densa, and the inner and outer less-dense electron layers referred to as the lamina lucida externa and interna, respectively [63]. The lamina lucida layers contain high levels of proteoglycans (hence they appear as clear or 'lucid' areas) that decorate a meshwork of small filaments that attach the lamina densa to the epithelial cell surface, and to the collagen fibrils within the stroma on the basal side. This creates a highly organized ECM layer between the epithelial cell and its underlying stroma. The major constituents of basal lamina are LN, collagen IV, nidogens, and perlecan.

Laminins LNs are large heterotrimeric glycoproteins (~900 kDa) found in at least 15 different combinations that are derived from five α, three β, and three γ subunits, coded from distinct genes [64]. Each LN chain includes globular, coiled coil, and rodlike regions [65]. The three distinct chains are intertwined at the coiled coil regions by disulfide bonds to form a cross-shape structure [65]. LN deposition appears to be restricted to the region of the basal lamina, is in direct physical contact with epithelial cells, and thus is likely critical mediators of epithelial cell-ECM interactions in vivo. Different LNs can be associated with specific tissue compartments or organs. For example, LN8 (α4, β1, γ1) is expressed by endothelial cells and contributes to the vascular basal lamina [15, 66], while LN10 (α5, β1, γ1) is associated with villus cells of the small intestine [67]. These and other examples suggest that particular LNs may be coupled with tissue-specific differentiation or function. In this review, we will focus on LN111 (α1, β1, γ1; known as LN1) and LN332 (α3, β3, γ2; previously known as LN5) due to their established presence in the mammary gland.

LN111 is one of the major constituents of the basal lamina and was the first LN characterized. LN111 was isolated from a poorly-differentiated murine sarcoma (Engelbreth-Holm-Swarm (EHS) sarcoma), and is a principal component of Matrigel, a commercially-available basement membrane substitute [68, 69]. LN111 is necessary for cell differentiation as documented in murine embryogenesis, where loss of any LN111 chain (α1, β1, or γ1) causes embryonic lethality due to improper basal lamina assembly and function [70, 71]. An insightful example was obtained after deletion of specific domains in the C-terminus of the LN α1 chain [9]. The C-terminus of the LN α1 chain consists of 5 globular domains with known cell binding motifs [72]. Deletion of the α1 globular 4–5 domains, which are binding sites for the ECM receptor dystroglycan, impeded pluripotent epiblast differentiation [9]. Interactions between α1 globular 4–5 domains and dystroglycan are crucial for gastrulation and for the formation of the primary germ layers, likely because LN is needed for epithelial cell polarization [9]. Using a 3D culture model, Bissell and colleagues showed that LN111-rich reconstituted basement membrane is pivotal for apicobasal polarization of mammary epithelial cells and for lumen formation of acini [73–75]. Cultured luminal mammary epithelial cells did not form acini in collagen I gels unless the cells were mixed with myoepithelial cells [76]. Petersen and colleagues identified myoepithelial cells as the primary source of LN111 via in vitro assays and through staining of human breast tissue [76]. The in vitro studies have demonstrated that LN111 instructs acinar formation, in part by facilitating deposition of a mammary-specific basal lamina, given that newly-synthesized LN332 and collagen IV are incorporated into a basal lamina-like structure in a 3D culture model in the presence of LN111 [77, 78].

LN332 is expressed by mammary epithelial cells and can induce adhesive contacts in epithelial cells via α6β4 or α3β1 integrins [79, 80]. Moreover, MMP2-cleaved LN332 fragments (from the γ2 chain) have been shown to bind the epidermal growth factor receptor (EGFR) [81]. LN332 fragment-EGFR interaction was associated with pro-migratory effects on mammary epithelial cells as well as downstream activation of EGFR signaling, although the results from the cell growth assay were inconclusive [81]. Further, by culturing freshly-isolated murine mammary epithelial organoids that included luminal epithelial and myoepithelial cells in a 3D culture model, Werb and colleagues demonstrated that the front edges of actively-elongating ducts were devoid of intact LN332 [80]. Moreover, LN332 fragments are present in the mammary gland during mid-pregnancy and involution, suggesting that LN332 contributes to the tissue remodeling that occurs during these stages [81, 82]. Using a pan anti-LN antibody,

LN fragments were apparent in mammary tissue during early involution in the rat [23]. Nevertheless, the functional consequences of LN turnover during involution are still unknown. While LNs experience turnover during early involution of the gland [23], LN transcript and total protein levels change only modestly with pregnancy, lactation and involution, suggesting a fundamental requirement for LNs in mammary epithelial maintenance, rather than for stage-specific functions.

Collagen IV Collagen IV is also a major component of the basal lamina and is thought to be its primary scaffold protein [16, 83]. Unlike fibrillar collagen, collagen IV is a network-forming collagen. It is a heterotrimer and can be comprised from six possible genetically distinct α chains. Collagen IV α chains trimerize to form three distinct 'protomers' that can be distributed in a tissue-specific manner [84]. Two collagen IV protomers bind through the C-terminal non-collagenous 1 (NC1) domain to form a hexamer [16]. Hexamers interact with one another through N-terminal 7 S domains to generate a tetramer [16]. Yurchenco and colleagues demonstrated that the lateral interactions among tetramers created a polygonal network of collagen IV and a 3D mesh [16, 85]. The $\alpha1.\alpha1.\alpha2$ protomer was suggested to form the initial sheet-like basal lamina during embryogenesis [84]. Disruption of the COL4 $\alpha1$ or $\alpha2$ locus in mice is embryonic lethal [84]. Surprisingly, in collagen IV-deficient mice, LN111 and nidogen 1 were deposited and organized into a basal lamina-like structure, during early embryogenesis (prior to E10.5). The basal lamina in these mice had a discontinuous and altered structure, suggesting that collagen IV is not required for LN111 and nidogen assembly but is required to stabilize the nascent basal lamina [84]. Furthermore, other collagen IV α chains did not compensate for loss of the $\alpha1.\alpha1.\alpha2$ protomer [84]. Later in development, the $\alpha1.\alpha1.\alpha2$ protomer is replaced with different collagen IV protomers such as $\alpha3.\alpha4.\alpha5$ in the glomerular basal lamina [86]. Evidence for the importance of protomer tissue specificity has been obtained from patients with Alports syndrome who have a mutation in the COL$\alpha5$ chain. This mutation leads to absence of the $\alpha3.\alpha4.\alpha5$ protomer (IV) based network in the glomerular basal lamina, impeding the required switch from the fetal $\alpha1.\alpha1.\alpha2$ protomer to the $\alpha3.\alpha4.\alpha5$ protomer (IV) [86]. Alports syndrome leads to progressive renal failure among other conditions [87].

Although it is unclear which α chains comprise collagen IV in the mammary gland, collagen IV deposition has been found necessary for proper formation of the mammary lamina densa. Disruption of collagen deposition in the basal lamina in vivo resulted in collapsed alveolar structures in proliferative mammary epithelium, resulting in a phenotype similar to that seen during early involution [88]. In another study, Kidwell and colleagues demonstrated that mammary epithelial cells preferentially attached to collagen IV verses collagen I in vitro [89]. Hence, collagen IV is essential for supporting basal lamina structure and provides an anchor for mammary epithelial cells, indicating its specific role in maintaining cell viability. On a different note, cryptic domains of type IV collagen fragments were demonstrated to function as anti-angiogenic factors. Tumstatin is a fragment from the C-terminus of collagen IV $\alpha3$ chain and inhibits angiogenesis by blocking $\alpha V\beta3$ integrin signaling [90, 91]. Further, during early to mid-involution, alveolar loss is associated with both basal lamina remodeling and a decrease in vascular support needed for lactation [90, 92]. Thus, there may be a link between basal lamina turnover and vascular regression that occurs during early to mid-involution.

Nidogen Nidogens (also known as entactins) are sulfated glycoproteins (150 kDa) synthesized by fibroblasts as in various tissues such as skin, lung, central nervous system, and limb [93–95]. There are two genes in the nidogen family that encode nidogen 1 and nidogen 2. Nidogens are incorporated into the basal lamina upon secretion [93, 96]. Germ layer cooperation in the assembly of basal lamina is highlighted by the fact that mesodermal-derived fibroblasts make nidogen and ectoderm/endoderm-derived epithelial cells produce LNs. Nidogen is found in a 1:1 stoichiometric ratio with LN and can be viewed as a stabilizing component of the basal lamina by serving as a bridge between LN111 and collagen IV [97]. For example, the globular domain G3 of nidogen 1 binds to the C-terminal short arm of a LN $\gamma1$ chain and the G2 domain of nidogen binds collagen IV [98, 99]. Nevertheless, deletion of nidogen 1 in mice demonstrated that it was not essential for basal lamina assembly [100], as nidogen 2 appeared to compensate [101]. Furthermore, mice lacking both nidogen 1 and nidogen 2 do not die during embryogenesis, indicating that nidogens are not necessary for basal lamina assembly in most organs. However, lungs from newborn mice deficient in both nidogens had small alveolar spaces, thicker septa, and a prenatal decrease in expression of basal lamina constituents such as LN $\gamma1$ chain, which recovered to control levels by the perinatal period [102]. In addition, the ability of type II pneumocytes to produce pulmonary surfactant may be impaired given that the expression of surfactant protein B was reduced in double knockout newborn mice [102]. Pulmonary surfactant is important in lowering the alveolar surface tension as well as maintaining alveolar structure. Taken together, these data suggest that nidogen 1 and nidogen 2 are needed for proper function of alveolar epithelial cells in the lung. Their absence in the lung may be the reason mice lacking both nidogens die shortly after birth [102]. Nidogens also support tissue-specific differentiation as nidogen 1 promotes the ability of

LN111 to induce β-casein expression by mammary epithelial cells in vitro [103]. Collectively, these and other data suggest a functional relevance for nidogens in a tissue-specific manner, but surprisingly, also indicate that nidogens are not essential for basal lamina formation during embryogenesis.

Perlecan Perlecan is a proteoglycan that consists of a 470 kDa core protein with covalently-linked heparan sulfate, a specific-class of GAG, which increases the molecular weight (MW) of perlecan to over 800 kDa [104]. Analysis of perlecan distribution during development revealed its expression in the basal lamina of liver, lung, the vascular system, and other tissues as well as in interstitial stroma such as the interterritorial ECM of articular cartilage [105]. Further, mammary epithelial cells have been shown to secrete heparan sulfate proteoglycan, suggesting epithelial cells as a possible source of perlecan [17]. Disruption of the gene encoding perlecan (Hspg2) caused embryonic lethality in a large portion of mice. Lethality occurs at E10.5 due to defective cephalic development and effusion of blood to the pericardium, which is thought to result from basal lamina deterioration [106, 107]. The remaining perlecan-null mice died perinatally with severe skeletal anomalies, presumably due to the role of perlecan in bone matrix (as described above) [106, 107]. Moreover, Fässler and colleagues observed discontinuous areas in the basal lamina in vivo that separated the neuroepithelium from the underlying mesenchyme [106]. All together, the phenotypes of the null mice suggest that perlecan is essential for basal lamina assembly in several organs and for bone matrix function. Additionally, perlecan's soluble C-terminal fragment, endorepellin, disrupted capillary morphogenesis in vitro by inhibiting endothelial cell migration, and angiogenesis [108]. Moreover, perlecan mediates the effects of several fibroblast growth factor (FGF) family members. Perlecan was suggested to interact with FGF2 through its core protein and GAG group [109]. These interactions can enhance the potency of FGF2 signaling through FGFR1 and FGFR3 in vitro and consequently have a pro-angiogenic effect [109, 110]. As with most ECM proteins, perlecan provides instructive cues dependent on context and structural integrity.

Intra- and Interlobular Stroma

Immediately adjacent to the basal lamina are 10–100 μm thick bands of organized, collagen-rich stroma, known as intralobular stroma, that surround individual alveoli (Fig. 2a, arrow in the insert box). Clusters of alveoli that compose a single lobule are further surrounded by bands of stroma referred to as interlobular stroma. Fibrillar collagens are the dominant structural constituents, with several other

ECM dictating intra- and interlobular stroma organization and functions.

Fibrillar Collagens Collagen I, III and V are the major fiber-forming collagens, where their individual proteins organize into bundles of various thicknesses and lengths to form the pink or blue fibrous material observed in H&E (Fig. 2a–c) and trichrome-stained (Fig. 2d–f) mammary sections, respectively. Collagen I has been demonstrated to be synthesized by fibroblasts, chondroblasts, osteoclasts, and odontoblasts during normal development and by activated fibroblasts during wound healing. Thus, mesenchymal cells are thought to be the primary source of collagen I. The collagen I precursor, procollagen (500–600 kDa) consists of two identical α1 (I)-chains and one α2 (I)-chain, each derived from a unique gene. Each α chain is made up of approximately 300 Gly-X-Y repeats where X is frequently proline, and Y is hydroxyproline [32, 111]. The Gly-X-Y repeats are essential for 'self-assembly' of collagen chains into a triple helix procollagen structure, where all the glycines face inward while the larger amino acids such as proline cover the outer positions [111, 112]. After assembly into the triple helix, peptidases remove non-helical registration peptides from the secreted procollagen [111] and subsequently it is transformed into insoluble 300 nm tropocollagen [111]. At the end of each triple helix tropocollagen molecule there are short non-helical telopeptides. These allow further stabilization and aggregation by covalent intra- and intermolecular crosslinking via LOX resulting in the basic fibrillar structure of collagen observed in histological sections [32, 111]. Based on its ubiquitous and abundant presence, fibrillar collagen I is considered the backbone of stroma, including that in the mammary gland. As such, the physical and biochemical properties of collagen I likely contribute significantly to the functional attributes of the mammary stroma.

Work from the Taylor-Papadimitriou and Sonnenschein labs demonstrated that collagen I supported mammary ductal formation in 3D culture [113, 114]. Further, when the collagen-specific α2 integrin subunit was knocked out in vivo, branching complexity in mammary glands was reduced [115]. Nonetheless, the epithelial layer in vivo is separated from the intralobular stromal ECM by the basal lamina. Therefore, how do these ECM subcompartments and stromal cells such as fibroblasts cooperate to influence mammary epithelial morphogenesis? Krause et al., embedded mammary epithelial cells with and without fibroblasts in collagen I gels and in Matrigel-collagen I gels [114]. Epithelial cells alone could only form ductal structures in collagen I gels in comparison to Matrigel-collagen I gels, however when co-cultured with fibroblasts, the epithelial cells formed ductal structures in both gel conditions [114]. Moreover, fibroblast-secreted factors such as hepatocyte

growth factor (HGF) can enhance the ability of mammary epithelial cells to form duct-like structures in collagen I gels [113]. Collectively, these studies demonstrate that interplays between fibrillar collagens, integrins, and growth factors are required for mammary duct development.

Fibrillar collagen-epithelial cell interactions are also critical for supporting the mammary epithelium during pregnancy and lactation. Collagen-associated receptor, DDR1 may control the tremendous increase in epithelial proliferation that occurs during pregnancy [8]. DDR1-null mice demonstartaed hyperproliferation of the mammary epithelium during pregnancy and were unable to lactate, despite the ability of luminal epithelial cells to express normal transcriptional levels of milk proteins [8]. Hence, interrupting the collagen-DDR1 interaction causes misguided instructive cues that are imperative for proper alveolar expansion and secretory function [8].

With cessation of milk secretion and weaning, the mammary epithelium involutes and returns toward its pre-pregnant, non-secretory state. The stroma in the involuting gland shares attributes with fibrotic, desmoplastic stroma seen during wound healing. During involution, fibrillar collagens are upregulated in the mammary gland and an influx of innate immune cells such as macrophages are observed [116–118]. Based on gene array data, the onset of involution is characterized by elevated expression of several fibrillar collagens including collagen I, III, and V as well as an increase in the expression of genes involved in ECM turnover [23, 52]. We and others have demonstrated that non-fibrillar denatured collagen can be a positive chemoattractant for macrophages in vitro [118]. Although it is unclear whether macrophages are necessary for collagen accumulation or remodeling of the mammary ECM during involution, monocyte chemoattractant protein 1 (MCP-1) increases in early to mid-involution [118]. The presence of MCP-1 was shown to be important for collagen fiber formation in a chemical-induced skin fibrosis model, as MCP-1 null mice were resistant to chemical-induced skin fibrosis, including fibrillar collagen deposition [119]. Further, the loss of MCP-1 was associated with a decrease in macrophages and other innate immune cells, in addition to an increase in the small leucine-rich proteoglycan (SLRP) decorin. The relationship between SLRP and collagen fibrillogenesis will be discussed later in this review. Collectively, the data suggest that fibrillar collagen-macrophage interactions may, in part, drive the tissue remodeling programs within the involuting mammary gland.

Another open question is whether unique functions are imparted by distinct fibrillar collagens with respect to collagen bundle formation, ECM turnover, and cell-collagen interactions. Although collagens III and V form oriented super-structures characteristic of fibrillar collagen, these collagens have features distinct from collagen I. For example, localized helix instability in collagen III can be caused by the higher content of glycine in α1 (III) chains [32]. This structural feature may cause an increase in the turnover rate of collagen III and subsequently the collagen III matricryptins may serve as potent chemoattractants for innate immune cells. Furthermore, collagen III is a homotrimer consisting of a single α chain, while collagen V is a heterotrimer composed of three different α chains [111]. These variations in primary structure likely cause additional changes in biochemical properties, as fibrillar collagen I, III, and V interact with one another to assemble into supramolecular collagen bundles [111]. The assembly of these bundles is further supported by short triple-helical fibril associated collagens with interrupted triple helices (FACIT) collagens such as collagen IX, which can function as crosslinking bridges [32, 111]. Interactions between fibrillar collagens and FACIT collagens may be important for mammary gland remodeling by dictating collagen organization and turnover.

In summary, the function and expression levels of fibrillar collagens are altered during the various stages of mammary gland development. These studies suggest unique roles during duct formation, alveolar expansion during pregnancy, as well as to the demoplastic-like microenvironment during involution, highlighting the dynamic role of fibrillar collagens in the mammary gland. However, even for ECM proteins as ubiquitous as fibrillar collagens, our basic understanding of both function and mechanism of action remains largely unknown.

Fibronectin FN is a large dimeric glycoprotein (~500 kDa) that mediates cell adhesion, migration, proliferation, and branching morphogenesis. FN is encoded by a highly-conserved single gene and its deletion causes embryonic lethality during gastrulation, emphasizing its essential function [120]. FN consists of three distinct repeating modules known as type I, II, and III domains [121]. Although FN protein is encoded by a single gene, there are several variant FN forms that arise due to alternative splicing [121, 122]. Specific examples are the spliced sites in the type III domain, termed extra domain (ED) EDA, EDB, and IIICS. Extracellular stimuli such as HGF have been shown to regulate splicing in vitro [122]. EDA and EDB have been implicated in wound healing, as their expression is undetectable in normal liver but is rapidly upregulated during injury [123]. In addition to its essential role in early embryogenesis, FN is an essential regulatory protein for salivary gland branching morphogenesis [124]. Yamada and colleagues blocked cleft formation and branching in the salivary gland ex vivo by downregulating FN expression in salivary epithelial cells via siRNA or by inhibiting FN-epithelial cell interaction via anti-FN anti-

body or antibodies against β1 integrin subunit [124]. Further, branching morphogenesis was rescued by adding exogenous FN to siRNA-treated glands [124]. These studies have clearly demonstrated that FN is required for salivary gland branching morphogenesis, although, the question of whether this requirement is FN variant-specific is unknown [124].

Ovarian steroids are suggested to regulate FN expression in the mammary gland [24]. Haslam and colleagues measured a three-fold increase in FN protein levels around mammary ducts between pre-puberty and sexual maturity [24]. Moreover, ovariectomy of sexually-mature mice resulted in a 70% decrease in the FN concentration in the mammary epithelium [24]. FN and the fibronectin receptor α5β1 integrin are dramatically downregulated during late-pregnancy and lactation, and then upregulated during mid-involution [23, 24]. Interestingly, the EDA isoform associated with pathological conditions in the liver is developmentally regulated in the rat mammary gland similar to total FN, suggesting a role for EDA within the normal mammary gland [23]. During the early stages of involution, elevated matrix metalloproteinase (MMP) activity correlated with higher levels of cleaved FN [23]. Additionally, FN fragments can induce MMP9 activity in mammary epithelial cells in vitro, suggesting a positive feedback loop between FN fragments and MMP activity [23]. This has implications for clearance of the secretory epithelium in involuting glands because FN fragments can induce apoptosis in epithelial cells [58].

Tenascins The tenascin (TN) family consists of 5 members: TN-C, TN-R, TN-W, TN-X, and TN-Y. TN-C was the first member of the TN family to be discovered, and is a six-armed glycoprotein with a 3D structure known as hexabrachion. Each arm contains N-terminal structural domains for self-assembly, followed by EGF-like repeats, alternative-spliced FN type III domains, and a C-terminal globular fibrinogen-homology domain [125]. The MW of TNs ranges between 180 and 300 kDa, which may depend on alternative splicing [125, 126]. TN is suggested to display anti-adhesive properties via disruption of the interactions between the transmembrane heparan sulfate receptor sydecan-4, α5β1 integrin receptor, and fibronectin [127, 128]. An anti-adhesive effect of TN-C on cells such as satellite cells has been shown to be context-dependent because other cells, for example, embryonic dorsal root ganglion explants, were able to spread, migrate, and grow on TN-C ex vivo [129]. Disruption of the TN-C gene in mice did not produce any visible abnormalities [130]. Although, Kusakabe and colleagues demonstrated neurological defects in TN-C null mice [131, 132]. Nonetheless, TN-C expression is associated with multiple tissues during embryogenesis and pathological conditions. In general, TN-

C expression is transient and largely restricted to embryogenesis, yet it is upregulated in breast cancer [125, 133]. Hence, TN-C has been described as an oncofetal protein. TN-C was also demonstrated to be overexpressed in skin during wound healing, particularly in the dermis–epithelial junction [134]. In the mammary gland, TN-C is transiently expressed in dense stroma surrounding the budding epithelium during rodent embryogenesis, with little to no expression observed in the quiescent adult gland [135]. TN-C expression is again upregulated during lactation and early postpartum involution [23, 136]. TN-C was found both in the milk of lactating mice and women [137, 138]. The function of TN during lactation is unclear, especially in light of fact that β-casein expression was inhibited in mammary epithelial cells cultured on reconstituted basement membrane containing exogenous TN-C [136].

Another TN family member that was identified in the mammary gland is TN-X [62]. TN-X has only a tribrachion structure and is prominently expressed in testis and muscle [139]. Burch and colleagues identified a connective tissue defect in TN-X-deficient patients that involved hyperextensible skin and joints, vascular fragility, and improper wound healing similar to Ehlers-Danlos syndrome [140]. These observations suggest that TN-X maintains tissue elasticity. During lactation the mammary epithelial cells can experience considerable stretch forces, and it is of interest to determine whether TN-X provides such a function at this time.

SPARC SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin) has been identified in the mammary gland by gene array and mass spectrometry based proteomics [52, 62]. SPARC is a small, 32 kDa secreted glycoprotein that mediates cell-matrix interactions [141]. SPARC contains a calcium-binding C-terminal extracellular module, follistatin-like module, and N-terminal acidic module [142, 143]. SPARC-null mice exhibit abnormal collagen fibril morphology [144]. Using picrosirius red staining, the effect of SPARC on collagen fibrillogenesis was evaluated in the dermis [144]. Compared to wild type mice, the dermis in SPARC-null mice contained less striated collagen fibers [144]. Sage and colleagues found that SPARC preferentially binds to ECM proteins such as collagen I and LN111, but not to collagen III [145]. They also demonstrated that SPARC displays an epitope-specific anti-adhesive function that causes cell rounding [145]. In contrast, SPARC was shown to positively regulate FN assembly and integrin-linked kinase activity, which resulted in FN-induced stress fiber formation in lung fibroblasts [146]. SPARC has been reported to be developmentally upregulated within the mammary gland during the transition between lactation and postpartum involution, correlating with increased levels of collagens

and FN [23, 118]. Future research focused on SPARC function in collagen fibrillogenesis and FN assembly in a dynamic organ such as the mammary gland is warranted.

Small Leucine-Rich Proteoglycan (SLRP)

SLRP family members are characterized by N-terminal cysteine-rich motifs and tandem leucine-rich repeats (LRR) in their core protein. The core protein of SLRP is 'decorated' with one or more GAG chains. The SLRP gene family was categorized into five different classes based on several factors such as gene homology, distinctive N-terminal cyteine-rich motifs, and characteristic GAG side chains [147]. SLRPs were initially thought to only provide structural support and participate in collagen fiber formation. However, additional biological functions for SLRPs were recently identified [147]. SLRPs can directly bind to cell surface receptors and to growth factors, implicating them in various signal transduction pathways and cellular processes [147]. We will focus below on decorin and biglycan from class I, as these SLRPs have been determined to be present in the mammary gland via gene array and proteomics in multiple studies [52, 62].

Decorin Decorin core protein (~38 kDa) is covalently linked with a single chondroitin sulfate (CS) or dermatan sulfate (DS) chain resulting in an overall MW of 90-140 kDa [148]. Decorin is a secreted protein and is expressed in various cell types such as fibroblasts and astrocytes [149, 150]. Although decorin-deficient mice experienced skin and lung fragility, possibly due to aberrant organization of fibrillar collagen, they are viable. Variability in the shape and size of collagen fibrils in these mice was observed compared to wild-type mice, as determined by EM of dermal collagen [151]. Based on these studies, decorin has been suggested to be pivotal for proper spatial alignment of stromal collagen fibers. Another function of decorin was demonstrated by Iozzo and colleagues, where decorin suppressed signaling through ErbB2 in breast cancer cells in vitro and in xenograft models [152]. They suggested that decorin may inhibit EGFR signaling by initiating EGFR internalization and degradation by caveolar endocytosis [153]. Angiogenesis was also negatively affected by decorin as it downregulated vascular endothelial growth factor expression in tumor cells [154]. When endothelial cells were cultured in media from decorin-expressing tumor cells, they failed to migrate or form capillary-like structures in vitro [154]. An additional role of decorin is to bind TGF-β1 and sequester its activity in vitro [155]. Given that decorin is crucial for proper fibrillar collagen organization, one question concerns the role of decorin in mediating tensional changes required for TGF-β1 activation as

discussed in previous sections [26]. Array data demonstrate that decorin is upregulated during mammary gland involution [52], where it likely contributes to the transient increase in collagen fibers [118].

Biglycan Biglycan is also a member of the SLRP class I family and is comprised of a core protein (~38 kDa) that is covalently attached to two GAG chains (chondroitin sulfate and/or dermatan sulfate) with an overall MW of 150–240 kDa [148]. Young and colleagues observed a reduction in bone formation and mass in biglycan-deficient mice [156]. Disruption of biglycan gene expression did not cause upregulation of decorin expression and vice versa [151, 156, 157], although both biglycan and decorin belong to the same SLRP subfamily [157]. These results suggest that decorin and biglycan have distinct, non-overlapping roles [156]. Schaefer et al. have tested the function of biglycan in maintaining renal tissue elasticity under pressure-induced injury caused by unilateral ureteral ligation [158]. They concluded that biglycan can induce elastic fiber-associated protein fibrillin 1 expression in renal fibroblasts and mesangial cells, which subsequently help to retain elastic properties of the damaged tissue [158]. Their data is especially intriguing when it is taken together with work from Salgado et al. who demonstrated dynamic expression and localization of decorin and biglycan in the mouse uterus during the estrous cycle and pregnancy, suggesting that it is hormonally-regulated [159, 160]. These studies raise the question of whether biglycan and decorin are also hormonally-regulated in the mammary gland and whether they influence the elastic properties of the gland during periods of expansion. Moreover, biglycan transcript abundance in the mammary gland increased four- to five-fold during the transition from lactation to involution [52, 118].

Importantly, several members of the class II SLRP family including lumican, mimecan, and prolargin have been identified in the mammary gland, as well as evidence for their differential expression across the reproductive cycle [52]. Future studies are required to investigate the function of the class II SLRP family in the mammary gland.

Fibrous Connective Tissue

The third structurally-distinct stroma in the mammary gland is the relatively acellular fibrous connective tissue, which is often found in large swaths (mm to cm range in the rat and human) and is characterized by the absence of epithelium and presence of fibroblasts, immune cells, and high fibrillar collagen content (Fig. 2a, solid arrow head). While the fibrous connective tissue is considered to be separate from the intra- and interlobular stroma, these

compartments share many of the same ECM proteins such as fibrillar collagens, FN, TN, SLRPs, and SPARC. However, a unique feature of fibrous connective tissue is the presence of elastic fibers.

Elastic Fibers Elastic fibers provide structural support and elasticity to various tissues. Elastic fibers are comprised of a 31 kDa secreted protein called microfibril-associated glycoprotein (MAGP) and another glycoprotein, the 350 kDa fibrillin protein that assembles into 10- to 12-nm microfibrils [31]. Microfibrils then associate with other elastic fiber constituents including elastin, fibulins, and proteoglycans. The process of organizing elastic fibers in the connective tissue begins with the formation of the MAGP/fibrillin microfibrils that align parallel to fibroblasts and serve as tracks for the deposition of tropoelastin (70 kDa), which is a soluble precursor of elastin. Tropoelastin is rich in hydrophobic amino acids and contains low amount of polar amino acids including several lysine derivatives. The lysine derivatives are imperative for covalent crosslinking between monomeric elastin via LOX [31]. Microfibrils are described as a scaffold that facilitates the alignment of soluble monomeric elastin to coalesce and organize into amorphous elastin [31]. As elastic fibers mature postnatally, elastin becomes the dominant component, with microfibrils being gradually displaced to the outer layer. The resultant fibers have physical properties that can withstand changes in the mechanical environment [161]. An additional constituent of elastic fibers is the fibulin family (50–200 kDa), which have calcium-binding sites and consist of I, II, III domains with a central segment that is composed of EGF-like modules. Fibulin 5 (66 kDa) displays strong calcium-dependent binding to tropoelastin, while it weakly binds to carboxyl-terminal of fibrillin 1 [162]. Further, fibulin 5 knockout mice were generated to analyze fibulin 5 function in elastic fiber formation in the dermis. These mice demonstrated a requirement for fibulin 5 in tropoelastin deposition and potentially for optimal LOX activity in vivo [162, 163]. Moreover, proteoglycans such as biglycan and decorin were implicated in elastogenesis by binding to tropoelastin, fibrillin 1, and MAGP [158, 164, 165]. While much is known about the functions of elastic fibers in other systems such as lung, skin, and vascular system, their function in the mammary gland is still elusive. Elastic fibers are anticipated to be particularly important in the lactating mammary gland as the tensile forces continuously change due to suckling, milk accumulation between nursing, and myoepithelial contraction on the secretory alveolar epithelial cells. Further research on the function and regulation of the fibrous connective tissue, including elastic fibers, is necessary given the correlations between higher mammographic density, tissue tension, and increased breast cancer risk [28, 166, 167].

Future Directions

Immune Cells and ECM Remodeling

The ECM of the mammary gland plays a role in the infiltration and activation of immune cells. We have previously characterized involution as a wound healing microenvironment with high levels of alternatively activated or M2 type macrophages [118]. How the immune cells are recruited and 'activated' still remains unclear. However, many of the proteins that make up the ECM can fragment into matrikines and matricryptins, which have been demonstrated to influence leukocyte infiltration in other systems. Fragments of collagen I, collagen IV, LN and nidogen-1 have all been shown to promote chemotaxis of monocytes and neutrophils within the interstitial tissue, but it remains unknown whether tissue-derived matrix fragments enter the blood stream to recruit immune cells or augment their motility upon arrival to the local tissue [118, 168–171]. Once in the mammary gland, macrophages and neutrophils secrete proteases including MMP9 and elastase that are able to breakdown the ECM [118, 172, 173]. Without the influx of macrophages or neutrophils and their crucial proteases, remodeling of the mammary tissue to its non-secretory postpartum state could potentially be delayed or incomplete. Werb and colleagues have evaluated the importance of secreted proteases during involution in the mammary gland by inhibiting a serine protease, plasma kallikrein. They demonstrated that inhibition of this mast cell-associated protease during involution caused an accumulation of fibrillar collagen and delayed repopulation of adipocytes, thus preventing the gland from returning to its pre-pregnant state [174]. Combined, these studies indicate that ECM turnover and immune cell function are highly linked, creating a feedback loop that is necessary to complete gland remodeling after lactation.

In addition to facilitating immune cell infiltration, ECM fragments may act as ligands to receptors present on leukocytes in the mammary gland. Fragments of biglycan, heparan sulfate, and hyaluronan have been shown to act as ligands for toll like receptor 4 (TLR4). TLRs are part of the pattern recognition receptor family expressed on the cell surface of innate immune cells and dendritic cells. When a ligand binds to a toll like receptor the immune cell becomes activated and/or secretes cytokines that can further activate cells of the adaptive immune system. Babelova et al. recently demonstrated that soluble biglycan binds to the TLR 2/4 on macrophages, stimulating them to synthesize and release interleukin-1β, a proinflammatory cytokine [175]. Additionally, heparan sulfate and hyaluronan have been shown to bind to the TLR4 on dendritic cells, resulting in dendritic cell maturation [176, 177]. Once mature, dendritic cells are able to activate the adaptive immune system, where adaptive

immune cells respond and migrate to the site of ECM remodeling. Consistent with a similar mechanism of adaptive immune cell activation due to ECM fragmentation in the mammary gland, the presence of B cells in the mammary gland during involution has been confirmed; however their role remains elusive [172]. The adaptive immune system is typically activated after the innate response, although B cells have been shown to infiltrate the gland during early to mid-involution, prior to the peak in macrophage recruitment [172]. The presence of B cells may indicate that cells of the adaptive immune system can be attracted to the gland, similar to the innate immune cells, by the presence of proteolytic ECM fragments. Interleukin-4 and fibronectin, which are both upregulated during involution, have been shown to stimulate motility of B cells in vitro, and may account for the increased presence of B cells in the mammary gland during involution [118, 178]. The infiltration of immune cells such as macrophages, neutrophils, mast cells, and B cells into the postpartum mammary gland during involution suggests both the innate and adaptive immune systems play an important role in returning the gland toward its pre-pregnant state. However, with limited research on the function of immune cells in the mammary gland, additional studies are necessary to determine the role of the ECM in immune cell activation, as well as the role for immune cells in restructuring the mammary gland in vivo.

Summary

The in situ analysis of mammary stroma by EM, cytological stains, and immunohistochemistry have provided snapshot views depicting the mammary stroma as diverse and highly complex. Cell culture studies using purified ECM components clearly demonstrate distinct, non-overlapping roles for ECM proteins with functions that range from induction of cell death to induction of a secretory phenotype. Studies using ECM preparations derived from tissues [EHS tumor and rat mammary glands] further demonstrate the complex and dynamic nature of the endogenous ECM. To further our understanding of the composition and function of ECM in the mammary gland, it is necessary to increase our ability to obtain better qualitative and quantitative ECM proteomes. Progress in proteomics is beginning to permit non-biased, high throughput, quantitative assessments of ECM proteomes. Such results will likely transform future in vitro experiments by permitting a systems approach to the study of mammary ECM-epithelial cell interactions.

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The Function of Microenvironment in Breast Cancer Dormancy

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Breast cancer recurrences due to "awakening" of dormant micrometastases and/or locoregional cancerous lesions underscore the clinical importance of deciphering the mechanisms of dormancy. Recent studies suggest that metastatic gene traits are not the only component driving recurrence, rather, interactions between cancer cells and their environment are also active contributors to this process. A „tumor suppressive" microenvironment may benefit a patient in the short term, yet a long term consequence could be residual tumor cells that have potential to result in relapse if the environment losses its suppressive attributes.

We are using differences between nulliparous and parous reproductive states to investigate „tumor suppressive" ECM microenvironments. Parity has been shown to reduce risk of mammary carcinoma in rodents and humans. Mammary ECM isolated from parous female rats may contribute to this protection by inhibiting mammary epithelial cell growth and motility compared to mammary ECM isolated from nulliparous rats (1). Additionally, in an orthotopic xenograft breast cancer model, a decrease in tumor growth is observed when breast cancer cells are injected into the mammary fat pad of parous mice compared to nulliparous mice. To further analyze „tumor suppressive" and „tumor supportive" ECM, we utilize an *in vitro* tumor dormancy model developed in Jeffery Green"s lab. In this model, murine mammary tumor D2.0R cells entered dormancy when cultured on reconstituted basement membrane proteins (Matrigel) (2). Further, Ann Chamber"s lab has shown that D2.0R cells formed dormant lesions at secondary sites *in vivo*, highlighting the role of the microenvironment *in vivo* in the regulation dormancy (3). Knowing that the nulliparous mammary microenvironment is conducive to tumor progression, we evaluated whether nulliparous rat mammary ECM prevented D2.0R cells from entering a dormant state. As predicted, D2.0R cells were "awakened" and formed large organoid structures in the presence of nulliparous mammary ECM. An increase in Ki67 positive cells and a decrease in juctional E-cadherin staining are also apparent in these organoids in comparison to organoids formed on Matrigel. Additionally, phospho-ERK1/2 levels were increased in D2.0R cells on nulliparous mammary ECM compared to Matrigel. Evidence that the increase in cell proliferation induced by nulliparous matrix is mediated in part through the ERK pathway is suggested by an approximate 40% decrease in average organoid size and concurrent decrease in BrdU incorporation when cultures are treated with a MEK1/2 inhibitor (U0126) compared to vehicle. Inhibition of ERK-mediated growth in breast cancer cells can upregulate Akt (4). We observed equal amounts of phospho-Akt (Ser473) staining in D2.0R cells in both control and treated groups, suggesting that ERK inhibition primarily targeted proliferation in this model. However, Akt anti-apoptotic activities may differ between dormant and proliferative cells. Whether mammary ECM from parous rats can induce dormancy in mammary epithelial tumor cells is under investigation. Our goal is to use this physiologically relevant system to identify the compositional and functional differences between „tumor suppressive" and „tumor supportive" ECM as a model to study dormancy at primary and distant sites. This model may provide additional insight into the clinical course of dormant breast cancer cells and how to target them.

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Mammary extracellular matrix proteins contribute to the 'protective effect' of parity and may provide insight into tumor cell dormancy

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Identifying the role of the microenvironment in tumor cell transition from a quiescent/non-motile state to a proliferative/invasive state may shed light on why some tumors remain dormant, while others do not. This study focuses on understanding the functional and compositional differences between „tumor supportive" and „tumor suppressive" extracellular matrix (ECM) as the underlying „soil" that influences tumor dormancy. While a „tumor suppressive" microenvironment may benefit a patient in the short term, a long-term consequence may be existence of residual tumor lesions that could cause progression and/or relapse if these suppressive attributes are lost.

To study a physiologically relevant „tumor suppressive" microenvironment, we focused on the parous mammary gland since parity has been shown to reduce risk of mammary cancer in rodents and humans. To support this observation, we injected breast tumor cells into parous and nulliparous mouse mammary fat pads, and observed a decrease in tumor growth in the parous glands. We have previously shown that mammary ECM isolated from parous female rodents (parous matrix) inhibits mammary epithelial cell ability to form branching structures *in vitro* compared to nulliparous matrix. Thus, we hypothesize that parous matrix will impede mammary tumor growth and invasiveness. An *in vitro* tumor dormancy assay developed by Barkan et al. (Cancer Research, 2008) was utilized to test whether nulliparous matrix was conducive to tumor progression, while parous matrix had suppressive attributes. Mammary tumor cells previously shown to exhibit a dormant phenotype in this *in vitro* assay (D2.0R cells) stayed dormant on Matrigel and were suppressed on parous matrices. Yet, when these cells were exposed to nulliparous matrices they were "awakened" and formed larger organoids. Moreover, D2.0R cells on Matrigel and parous matrices had greater retention of epithelial features including increased junctional β-catenin and decreased cytoplasmic E-Cadherin. Phospho-ERK1/2 levels were also decreased in D2.0R cells on Matrigel and parous matrices. Additionally, a 40% decrease in organoid size was observed when D2.0R cells on nulliparous matrix were treated with a MEK1/2 inhibitor, suggesting that ERK signaling may be involved in activating these cells. Finally when breast tumor cells were co-injected into mouse mammary fat pads with either parous or nulliparous matrices, tumor growth was significantly reduced in the glands injected with tumor cells plus parous matrices. To address what may account for these functional differences, the composition of nulliparous and parous matrices is being evaluated via mass spectrometry-based proteomic analysis.

Our goal is to identify differences in the ECM components of „tumor suppressive" and „tumor supportive" microenvironments to gain mechanistic insight into the clinical course of dormant breast tumor cells and how to target them.

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Mammary Extracellular Matrix Proteins Contribute to the 'Protective Effect' of Parity and May Provide Insight into Tumor Cell Dormancy

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In breast cancer, therapy-resistant tumor cells can remain dormant for decades. While a „tumor suppressive‟ microenvironment may benefit a patient in the short term, a long-term consequence could be relapse if the suppressive attributes are lost. Recurrence has been reported to occur in about 20% of patients a full ten years or more after initial treatment, suggesting that loss of dormancy contributes to disease progression in these patients. The study of tumor dormancy has been hindered significantly because of the small size of dormant lesions, which exist *in vivo* as single cells or small clusters of cells. Dormancy is thought to be mediated by a „tumor suppressive‟ microenvironment, but the study of the dormant microenvironment is technically challenging, and little is currently known. In this study, we focus on understanding the functional and compositional differences between „tumor supportive‟ and „tumor suppressive‟ extracellular matrix (ECM) proteins as the underlying „soil‟ that influences breast cancer cell dormancy.

To model a „tumor suppressive‟ microenvironment, we focused on the physiologically relevant parous mammary gland since parity has been shown to reduce risk of mammary cancer in rodents and humans. Our rational is that by identifying suppressive ECM proteins at the primary site, i.e. the mammary gland, we may identify ECM proteins that contribute to dormancy at distant sites as well.

To investigate whether the parous mammary microenvironment is tumor suppressive, human breast tumor MCFDCIS.com cells were injected into age-matched parous and nulliparous mouse mammary fat pads. A decrease in tumor growth was observed in the parous glands. To investigate the role of the mammary ECM in mediating this suppression, breast tumor cells were co-injected into mouse mammary fat pads with mammary ECM isolated from either parous or nulliparous animals. Tumor growth was significantly reduced in the glands injected with tumor cells mixed with parous mammary ECM compared to nulliparous mammary ECM. An *in vitro* tumor dormancy assay developed by Barkan et al. (Cancer Research, 2008) was utilized to test whether nulliparous matrix was conducive to tumor progression, while parous matrix had suppressive attributes. Mammary tumor cells previously shown to exhibit a dormant phenotype in this *in vitro* assay (D2.0R cells) were suppressed in parous matrices. Yet, when these cells were exposed to nulliparous matrices they were "awakened" and formed larger organoids. Moreover, adherens junctions of D2.0R cells in parous matrices were less disrupted compared to these cells in nulliparous matrices including increased junctional β-catenin and decreased cytoplasmic E-Cadherin fragment. Phospho-ERK1/2 levels were also decreased in D2.0R cells in parous matrices. Additionally, a 40% decrease in organoid size was observed when D2.0R cells in nulliparous matrices were treated with a MEK1/2 inhibitor, implicating the ERK pathway in loss of dormancy. To address what may account for these functional differences, the composition of nulliparous and parous ECM is being evaluated via mass spectrometry-based proteomic analysis. Our goal is to identify differences in the ECM components of „tumor suppressive‟ and „tumor supportive‟ microenvironments to gain mechanistic insight into the clinical course and treatment of dormant breast tumor cells. Supported by grant #BC095850 from the Department of Defense.